Food uptake, lipid transport and vitellogenesis in the sea anemone *Nematostella vectensis*

Marion Lebouvier

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

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Contents

Abstract

Balancing energy input and output is crucial for the survival of all organisms, and involves the coordination of many physiological processes such as food uptake, nutrient storage, reproduction and growth. The uptake of food particles through endocytic mechanisms (e.g. phagocytosis, receptor-mediated endocytosis) is broadly observed and likely the ancestral mode of feeding in metazoans. However, only little is known about the biology and evolution of endocytic cell types involved in animal nutrition. Similarly, the dynamics and molecular pathways underlying the transport of nutrients is poorly investigated in animals without a circulatory system. The lack of available studies, especially in non-bilaterian animals (e.g. cnidarians, sponges) leaves a number of key questions unresolved: how did endocytic cell types evolve? What are the ancestral modalities of nutrient transport in animals? In my thesis, I address these questions by investigating the cells and molecular pathways underlying food uptake and, as a specific example of lipid transport, vitellogenesis, in the sea anemone *Nematostella vectensis*. By characterizing the path of food particles and dietary lipids from their ingestion to their incorporation into yolk, I aim to fill in gaps in our understanding of nutrient uptake and transport in non-bilaterians and thereby shed light on the evolution of these processes in animals.

In *Nematostella*, nutrient acquisition starts with the extracellular digestion of prey in the gastric cavity through secreted digestive enzymes. Using single-cell RNA sequencing (scRNA-seq), I characterized the cellular composition of the gastrodermal folds lining the gastric cavity (mesenteries) and found a high diversity of specialized gland cells expressing specific enzymatic repertoires. Extra-cellular digestion is followed by endocytosis and subsequent intracellular digestion of food particles. By using particle uptake assays, I revealed that phagocytosis and receptor-mediated endocytosis predominantly occur in specific regions of the mesenteries in *Nematostella*, highlighting a surprising regionalization of the anthozoan gastrodermis. These regions colocalize with the cellular expression of *Nematostella* orthologs of bilaterian genes typically involved in endocytosis (e.g. *mannose receptor*, *clathrin*). This strongly supports the digestive function of these cells and indicates a conserved nature of endocytic molecular

pathways between cnidarians and bilaterians. These results were further validated by scRNA-seq, which revealed three distinct populations of trophic endocytes co-localizing within the endocytic region of the mesentery.

In bilaterians, dietary nutrients are most often transported towards other tissues via the circulatory system in order to be stored or to support the metabolism of peripheral tissues. Cnidarians lack a circulatory system, and the gastro-vascular cavity is thought to distribute nutrients throughout the body. The extracellular matrix (mesoglea) was previously proposed to participate in nutrient transport, but its role in this process has so far been unclear. In the present work, I describe for the first time the dynamic trans-epithelial transport of lipids from the gastric cavity into maturing oocytes located in the mesoglea in a cnidarian. Consistent with their function in shuttling lipids between the gastric cavity and the oocyte, somatic cells of the gonad epithelium also produce the glycolipoprotein Vitellogenin, a conserved yolk precursor. Gene expression data shows that the uptake of Vitellogenin into growing oocytes likely occurs through receptor-mediated endocytosis using orthologs of the *vldlr*/*apolipophorin receptor* gene family. This supports the hypothesis that a specific Vitellogenin ligand/receptor pair is highly conserved in vitellogenesis between cnidarians and bilaterians. Finally, I characterized the expression and protein localization of ApoB, a *Nematostella* ortholog of the bilaterian systemic lipid transport proteins Apolipoprotein-B/Apolipophorins. ApoB protein was not detected in growing oocytes in *Nematostella* but surprisingly localized in spermaries, suggesting a role during spermatogenesis. Overall, these results demonstrate the mesogleal transport of lipids potentially using conserved lipoprotein-lipoprotein receptor pairs in the absence of a circulatory system, and raise the possibility of a rudimentary systemic lipid transport system in *Nematostella*.

Altogether, my thesis revealed that nutrient uptake in *Nematostella* involves a remarkable diversity of specialized cell types that define functional domains in the mesenteries. The molecular machinery for food uptake, intracellular digestion and lipid transport seems to be highly conserved between *Nematostella* and bilaterians, providing an opportunity to elucidate the ancestral state of mechanisms underlying energy homeostasis in the last common ancestor to cnidarians and bilaterians.

List of publications

Paper 1

Lebouvier, M., Steinmetz P.R.H. (in preparation). Food uptake and intracellular digestion occur via conserved endocytic pathways in specialized regions of the gastrodermis in *Nematostella vectensis*.

Paper 2

Lebouvier, M., Miramón-Puértolas P., Steinmetz, P.R.H. (in preparation). Conserved lipoprotein-LDL receptor pair potentially mediates lipid transport during vitellogenesis in the sea anemone *Nematostella vectensis*.

Paper 3

Lebouvier, M., Miramón-Puértolas P., Saudemont, B., Loe-Mie, Y., Marlow, H., Steinmetz, P.R.H. (in preparation). Single-cell atlas of the *Nematostella vectensis* mesentery uncovers a diversity of digestive cells and their transcriptional profiles.

Chapter 1: General introduction

1. Energy homeostasis in animals

All living organisms, whether bacteria, plants, fungi or animals, are characterized by their own stable internal environment. When this concept of constant *milieu intérieur* was first proposed by the physiologist Claude Bernard in the 19th century, it was introduced as the defining feature of "higher" organisms (i.e. warm-blooded vertebrates), life liberated from the external environment (Bernard 1878). However, far from being isolated systems, all organisms dynamically interact with their surroundings and constantly adjust their physiology in response to environmental perturbations. These adjustments maintain an equilibrium kept within a narrow window favorable to life by coordinated control mechanisms, which was termed in continuity with Bernard's work by Walter B. Cannon who coined the term "homeostasis" (from the Greek homeo: similar and stasis: state) (Cannon 1929). Many physiological parameters contribute to the maintenance of homeostasis. These can act on the systemic level, such as body temperature or oxygen content in the blood of animals, or on the much smaller cellular level, such as cell volume or intracellular ion concentrations (Cannon 1929; Romero 2004). Deviations from the homeostatic balance result in immediate physiological or behavioral reactions. For example, a drop in body temperature in a mobile animal triggers a move towards a warmer micro-environment. A change in intracellular pH leads to the activation of cell-membrane ion pumps that help re-establishing homeostatic levels. Ultimately, maintaining homeostasis is an essential condition of life and the driver of many physiological processes and behaviors, making it a fascinating topic for physiologists and evolutionary biologists alike.

Energy homeostasis, the balance between energy intake and expenditure, involves a particularly large array of physiological processes in animals. On one hand, energy is acquired through food consumption, and often stored in specialized tissues in the form of nutrient reserves to be used in periods of high energy demand or food scarcity (Keesey and Powley 2008). On the other hand, the energy derived from dietary nutrients is essential for metabolism, growth, reproduction and motility (Hill et al. 2016).

Manipulating energy intake and outputs can therefore have major consequences on the metabolism, physiology and life cycle of animals. Lowering caloric intake has been linked to an increased lifespan in many organisms, including mice, nematodes, flies and even primates (Masoro 2009; Kapahi et al. 2017; Pifferi et al. 2018). Body size can also be affected by nutrient availability as some animals, such as planarians or jellyfish, reduce dramatically the number of body cells in response to starvation and regrow to a normal size when food is available again (Lilley et al. 2014; Felix et al. 2019; Fujita et al. 2019). As the production of gametes is a very energy consuming process, reproductive success in animals is tightly linked to food availability (Eckelbarger 1994; Hahn et al. 2005; Tixier et al. 2015; Alqurashi et al. 2020). In many marine species such as calanoid copepods, egg production follows food intake by only a few hours (Eckelbarger 1994) while in *Drosophila*, a change in the quality of the diet leads to a 60-fold decrease in egg production (Drummond-Barbosa and Spradling 2001).

The regulation of energy homeostasis at the intracellular level relies on the ancient TOR (Target of Rapamycin) pathway, which is thought to have evolved prior to the last eukaryotic common ancestor (LECA) (Van Dam et al. 2011). However, the emergence of multicellularity and complex body plans required the TOR pathway to integrate extra-cellular signals to ensure a systemic intercellular coordination between tissues and organs (e.g. vertebrate brain-gut axis; Scarlett and Schwartz 2015). Numerous studies on bilaterian research organisms have revealed complex networks of endocrine signals conveyed by the circulatory system that regulate food uptake, nutrient storage and the use of dietary resources. One of the most extensively studied regulatory mechanisms is the Insulin signaling pathway which is involved in the metabolism of glucose and lipids as well as in the regulation of growth and reproduction in insects, nematodes and mammals (Kimura 1997; Garofalo 2002; De Meyts 2004; Acevedo et al. 2007). In another example, the hormone leptin regulates food intake in mammals and also interferes with oocyte maturation (Pérez-Pérez et al. 2015).

During evolution, animals have adopted various strategies to balance their energy intake and consumption, prioritizing physiological strategies (e.g. promoting growth over reproduction) and developing a wide array of control mechanisms that preserve the

stability of their internal environment. In order to elucidate the evolution of energy homeostasis, we must get an understanding of the physiological and molecular processes at play on both sides of this equation: how do organisms obtain energy, and how do they spend their resources?

2. How to obtain energy: digestion in bilaterians

When the homeostatic balance is disturbed and an animal's energy stores (e.g. lipids in adipose tissues) need to be replenished, regulatory mechanisms induce the onset of feeding behaviors which ensure that the organism obtains the energy it needs to stay alive and reproduce by ingesting food (Woods et al. 1998; Hill et al. 2016). In order to become available as fuel for the body, food is progressively broken down in the gut through digestion. This process is therefore a critical step in the regulation of energy homeostasis, and in the first part of this work, I will focus on its underlying cellular and molecular mechanisms.

2.1 Animal digestive systems: from simple cavity to regionalized through-gut

Animals display a great diversity of digestive tracts, a trait that influences the modalities and efficiency of digestion. Yet, interestingly, the evolution of bilaterian gut morphologies is still a strongly debated topic. While it is commonly believed that a blind gut with only one opening was present in the last common ancestor (LCA) of bilaterians and their sister group, the cnidarians, it is still unclear how and when the transition to a through-gut occurred (Arendt and Nübler-Jung 1997; Martindale and Hejnol 2009; Hejnol and Martín-Durán 2015). Two main evolutionary scenarios have been proposed to explain the emergence of a second body opening, and therefore the formation of a through-gut: (1) an ancestral single gastric opening becomes the new mouth or anus, a second opening is newly acquired (Meinhardt 2004; Martindale and Hejnol 2009; Presnell et al. 2016) (2) an ancestral slit-like body opening fuses along the midline and gives rise to a mouth on one end and an anus on the opposite end of the initially single opening (Arendt and Nübler-Jung 1997; Steinmetz et al. 2007; Nielsen et al. 2018).

Among bilaterians, a sack-like blind gut with a single opening is mainly found in xenacoelomorphs, spiralians (e.g. planarians) and in some echinoderms (Schmidt-

Rhaesa 2007; Hejnol and Martín-Durán 2015). This morphology does not allow for a strong regionalization of the gut, although it can be subdivided or branched in some groups such as platyhelminths (Brusca et al. 2016). A segmented through-gut, with an anterior mouth and a posterior anus, is more widely distributed among bilaterian taxa (e.g. in all deuterostome and ecdysozoan groups) and the segmentation into specialized gut regions is believed to have evolved several times convergently (Schmidt-Rhaesa 2007). Segmented through-guts are usually subdivided into three distinct regions: (1) an anterior foregut, often associated with accessory structures contributing to the mechanical and chemical breakdown of food (e.g. stomach in vertebrates, crop in insects); (2) a midgut, involved in further enzymatic digestion and in the absorption of nutrients; (3) a posterior hindgut where water is reabsorbed and residual products are processed before being discharged (Hejnol and Martín-Durán 2015). This type of digestive system offers the advantage of a unidirectional digestion where the food goes successively through different highly specialized regions, allowing a constant food uptake and increasing the efficiency of the digestive process and nutrient absorption (Schmidt-Rhaesa 2007; Hejnol and Martín-Durán 2015).

2.2 Extracellular digestion in the lumen of the gut

All bilaterians, independently of their gut morphology, are able to break down food outside the cells of the gut, a process called extracellular digestion (Yonge 1937; Hill et al. 2016; Steinmetz 2019). In species possessing a complex through-gut such as insects or vertebrates, extracellular digestion is often so efficient that it is sufficient to break down food into assimilable nutrient monomers (e.g. amino acids, mono-saccharides) (Karasov and Hume 2011; Lemaitre and Miguel-Aliaga 2013; Hill et al. 2016; Steinmetz 2019). This process occurs through the activity of digestive enzymes, which are most often secreted into the lumen of the digestive tract or are bound to the apical membrane of the epithelial cells lining the gut (Hooton et al. 2015; Hill et al. 2016; Miguel-Aliaga et al. 2018).

Extracellular digestive enzymes of the digestive tract are produced by a variety of cells and show a very strong substrate specificity. In mammals, the first steps of extracellular

digestion occur in the stomach where food is broken down by its acidic environment and the activity of gastric lipases and peptidases (Hooton et al. 2015). In addition, pancreatic cells and enterocytes of the midgut play a central role in the high efficiency of extracellular digestion in vertebrates. The pancreas is the main producer of amylases (breaking down starches), lipases (e.g. pancreatic lipases, phospholipases), exopeptidases (e.g. carboxypeptidases) and endopeptidases (e.g. trypsins, chymotrypsins) (Karasov and Hume 2011; Hill et al. 2016). These enzymes are discharged in the anterior portion of the midgut, often in an inactive form as to avoid auto-digestion of the digestive tract, and are complemented by the enzymatic production of intestinal enterocytes. These columnar cells of the midgut epithelium present a high number of microvilli on their apical membrane and have a dual function during digestion: extracellular digestion and absorption. They play an important role in the production of extracellular digestive enzymes (e.g. sucrase, dipeptidases) that either remain intimately linked to the cell membrane or are released into the lumen of the gut, and additionally take up the digested assimilable nutrients and transfer them to the circulatory system (Hooton et al. 2015; Hill et al. 2016). The combined action of the different parts of the gut thus allows for a progressive and efficient degradation of food as it moves through the digestive system. Some variations on this theme exist: for example, many species of fish (e.g. lamprey, hagfish) do not possess a pancreas, but rather isolated or small groups of exocrine cells intercalated in the intestinal epithelium that are homologous to the exocrine pancreas of most other vertebrates (Youson and Al-Mahrouki 1999). In animals with a blind gut, digestive enzymes are secreted by epithelial cells into the lumen of the gastrovascular cavity where extracellular digestion occurs (Brusca et al. 2016). This system does not allow for a unidirectional movement of food and the successive digestion and absorption of specific nutrients, and therefore functions less efficiently than a through-gut.

Interestingly, the diversity of animal diets is mirrored by a diversity of specialized digestive enzyme repertoires. For example, a study conducted in bats showed that an evolutionary shift in the diet from insectivory to frugivory resulted in an up to 15-fold increase in sucrase activity in the intestine of the fruit-eating species (Schondube et al. 2001). Similarly, the analysis of intestinal enzymatic activity in a group of freshwater

fishes with diverse diets revealed that carnivorous species had a higher chitinase activity compared to herbivorous species (German et al. 2010).

As a summary, the through-gut of most bilaterians is extremely efficient at digesting food extracellularly. However, in those possessing a less complex digestive system, extracellular digestion often breaks down food into smaller particles and macromolecules rather than into nutrient monomers. In these species, extracellular digestion therefore represents a first step in a digestive process that is ultimately completed inside the cells of the digestive tract.

2.3 Intracellular digestion, an ancestral feeding mode

Intracellular digestion involves the internalization of food particles or macromolecules by digestive cells, often via specific receptors, and their degradation in the lysosome by intracellular digestive enzymes (Pollard et al. 2017; Rosales and Uribe-Querol 2017). Cellular uptake occurs through three main pathways collectively termed endocytosis: phagocytosis, micropinocytosis and macropinocytosis (Pollard et al. 2017). These mechanisms of particle internalization as well as the underlying molecular pathways are widely conserved among eukaryotes including in protists closely related to animals (e.g. choanoflagellates), suggesting that they represent the ancestral mode of digestion in animals (Desjardins et al. 2005; McMahon and Boucrot 2011; Pollard et al. 2017). Given my interest in the evolutionary aspects of intracellular digestion, this thesis will accordingly focus on endocytic particle uptake mechanisms.

2.3.1 Uptake of larger particles via phagocytosis

Phagocytosis is a receptor-dependent mechanism that mediates the specific uptake of large (>0.5µm) particles (Figure 1A) (Underhill and Goodridge 2012; Gray and Botelho 2017; Lancaster et al. 2019). In a first step, the cargo is detected by receptors located at the membrane of phagocytic cells (Gray and Botelho 2017). These receptors recognize molecules located at the surface of the particles and are usually able to bind a range of substrates (Ofek et al. 1995). Receptors that directly recognize the target are termed non-opsonic, and include C-type lectin receptors (e.g. Mannose receptor) and scavenger

receptors (e.g. CD36/Fatty Acids Translocase). Another group of receptors involved in phagocytosis are opsonic, meaning that they recognize molecules present on the surface of particles. The most notable example is the Fcγ receptor family, that recognizes antibodies attached to pathogens (Freeman and Grinstein 2014; Baron et al. 2016; Rosales and Uribe-Querol 2017). After the cargo is bound to the phagocyte, intracellular actin remodeling leads to the formation of a dip in the cell membrane: the phagocytic cup (Figure $1A_1$). From this region pseudopods extend progressively (Figure $1A_2$) and engulf the particle, finally leading to the formation of a phagosome, a vesicle that buds off into the cytoplasm (Figure $1A_3$) (Swanson et al. 1999; Rougerie et al. 2013). Cell membrane and actin cytoskeleton remodeling is mediated by a network of interacting proteins highly conserved between fungi and vertebrates. Key components include Engulfment and cell motility (ELMO), small GTPases of the Rho family as well as Wiskott-Aldrich Syndrome Proteins (WASPs) and Actin-related proteins (Arps) (Gumienny et al. 2001; Niedergang and Chavrier 2005; Rougerie et al. 2013). While the actin-mediated reshaping of the cell membrane is essential to the formation of a phagocytic cup, it is important to note that cytoskeleton remodeling as such is not endocytosis-specific. Most genes involved in phagosome formation are also involved in other cell membrane remodeling processes including cell migration (Gumienny et al. 2001) and cell division (Dutartre et al. 1996; Yoshizaki et al. 2003).

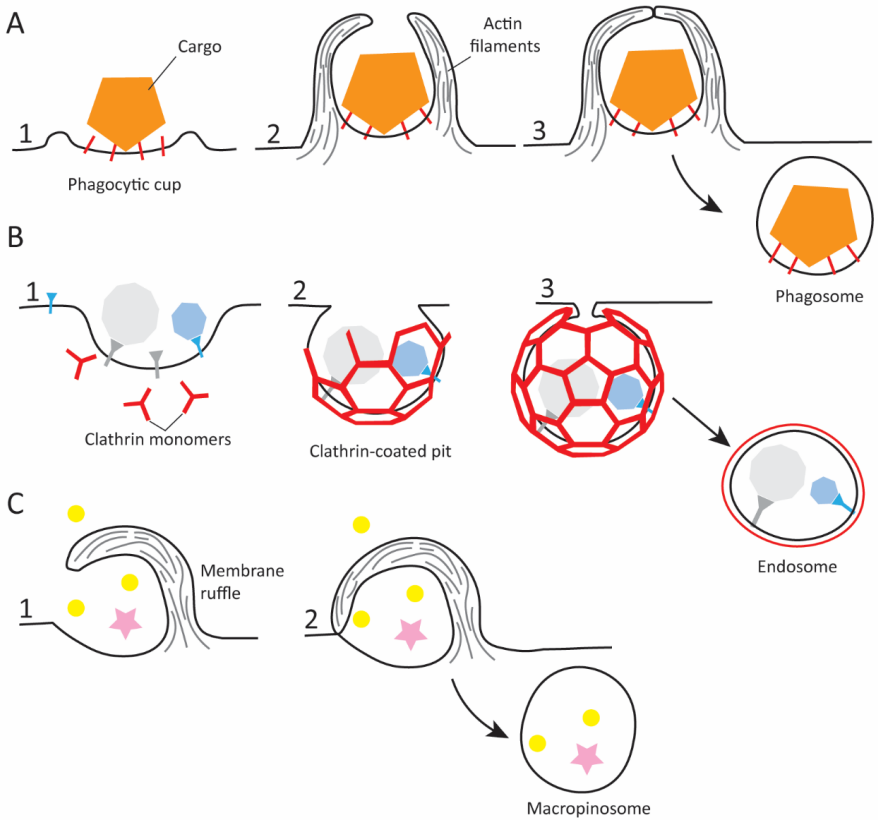

Figure 1: The three main cellular particle uptake mechanisms. (A) Schematic representation of phagocytosis. A large particle binds to membrane receptors on the surface of a phagocytic cell, triggering the formation of a phagocytic cup (1). Through actin-mediated membrane remodeling, pseudopods engulf the particle (2), fuse and a phagosome buds into the cytoplasm (3). **(B)** Schematic representation of clathrin-mediated endocytosis. As small particles and macromolecules bind to specific membrane receptors (1), clathrins (in red) form a coat around the endocytic pit (2). When the coat is complete, the vesicle is pinched off and released into the cytoplasm (3). **(C)** Schematic representation of macropinocytosis. Actin-mediated membrane ruffling induces the formation of protrusions that in some cases close to form a macropinosome, engulfing extracellular medium and the particles within. Note that the schematics are not to scale: approximate size of intracellular vesicles are 0.1-10µm for phagosomes, 100-150nm for clathrin-coated vesicles, 50-1000 nm for macropinosomes (Kerr and Teasdale 2009; Pollard et al. 2017).

2.3.2 Micropinocytosis/Receptor-mediated endocytosis: specific uptake of small food particles

The internalization of smaller (<0.5µm) particles and macromolecules can occur in a specific manner via receptor-mediated endocytosis, a process also commonly known as micropinocytosis. Currently, three different micropinocytosis uptake modalities have been described: clathrin-dependent, caveolin-dependent, and clathrin- and caveolin-independent endocytosis (Mayor and Pagano 2007; Pollard et al. 2017).

Clathrin-mediated endocytosis (Figure 1B) is the main micropinocytic pathway involved in the uptake of food. The first step of this process consists in the recognition of target molecules by receptors located in the membrane of the endocytic cell (e.g. low density lipoprotein receptors/LDLRs, transferrin receptors). Receptor binding initiates the recruitment of cargo-specific adaptor proteins, that in turn recruit clathrins, triskelion-shaped scaffolding proteins (Fig.$1B_1$). Clathrin polymerization leads to the formation of a clathrin-coated pit in the membrane (Fig.$1B_2$), which progressively invaginates until an endocytic vesicle is pinched off and released into the cytoplasm (Fig.$2B_3$) (McMahon and Boucrot 2011; Pollard et al. 2017). Shortly after their formation, clathrin covered vesicles are uncoated and the scaffolding proteins are recycled by the cell (McMahon and Boucrot 2011).

The caveolin-dependent endocytic pathway has not been linked to the uptake of food particles, but contributes to the systemic distribution of nutrients in some bilaterians. Caveolin proteins form a scaffold around nanoscopic invaginations found mainly on the surface of endothelial cells. These pits contain receptors that bind to their respective cargo, and bud into the cytoplasm, allowing the transfer of serum proteins and nutrients from the circulatory system into the different tissues of the body (Pollard et al. 2017). The clathrin- and caveolin-independent pathway is the least understood micropinocytic pathway and has not been found to play a role in nutrition-related processes. It mediates the internalization of a wide array of cargoes including interleukins, cadherins and toxins (Mayor and Pagano 2007; Pollard et al. 2017).

2.3.3 Non-specific particle uptake via macropinocytosis

Contrary to phagocytosis and micropinocytosis, macropinocytosis is a receptor-independent mechanism. Sometimes called ‘cell drinking’, it consists of a non-specific ingestion of small particles and macromolecules with extracellular fluid (Figure 1C) (Kerr and Teasdale 2009; King and Kay 2019). Macropinosomes derive from plasmatic ruffles, actin-driven protrusions of the membrane that occur constitutively or are induced by signaling molecules (Fig.1C_1). Ruffles can constrict and fuse, enclosing a drop of extracellular medium and the particulate matter that it may contain (Fig.1C_2) (Swanson and Watts 1995).

2.3.4 Degradation of the cargo in the endosome/lysosome system

After internalization, the digestion of food particles and macromolecules is finalized inside the cell as endocytic vesicles are channeled into the endosome/lysosome system.

Shortly after their formation, endocytic vesicles fuse with early endosomes (EE), which are vesicular structures located at the periphery of the cells (Figure 2). In EEs, the cargo, receptors and other membrane proteins of the endocytic vesicle are sorted out, leading to the recycling of some of these components back towards the cell membrane (Huotari and Helenius 2011; Repnik et al. 2013). Through homotypic fusions and contributions of vesicles from the trans-Golgi network, the EE grows in size and starts accumulating lysosomal enzymes (Figure 2). Some of these enzymes are already active in the EE, and more will become active during the maturation process as the pH progressively decreases (Huotari and Helenius 2011; Pollard et al. 2017). The transition from EE to late endosome (LE) is accompanied by the migration of the organelles from the periphery of the cell towards the peri-nuclear region, and is mediated by GTPases of the Rab family (Huotari and Helenius 2011). Rab5 is a major marker of EEs, and mediates the specific fusion between EEs and with endocytic vesicles (Christoforidis et al. 1999). The transition to late endosome (LE) occurs through a ‘Rab switch’: Rab5 is lost and replaced by Rab7, which participates in the last step of the intracellular digestion, the fusion of the LE with a lysosome (Rink et al. 2005; Hyttinen et al. 2013).

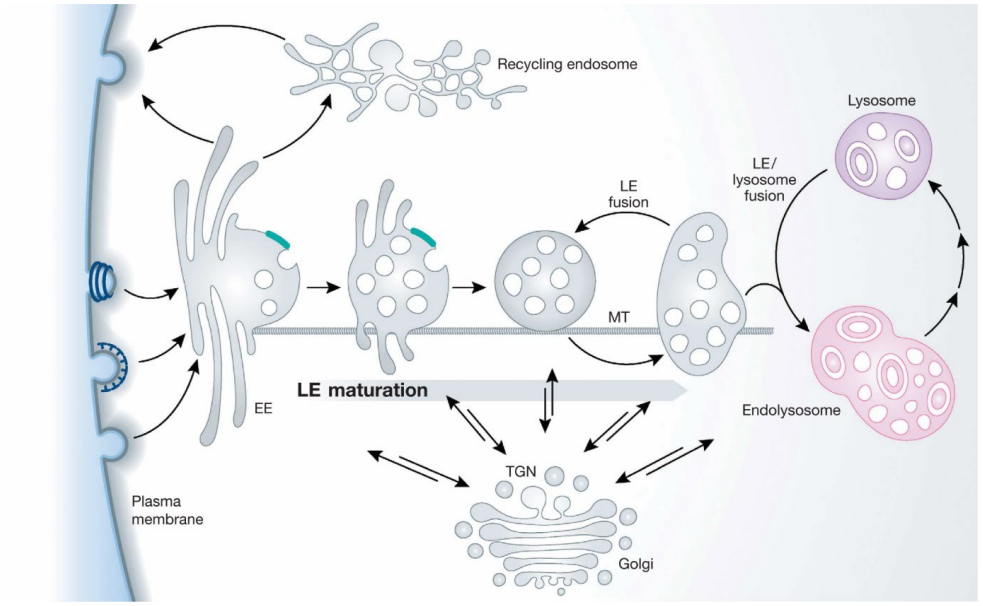

Figure 2: The endosome/lysosome system. Schematic representation of the successive fusions and transformations undergone by endocytic vesicles in order to complete intracellular digestion. Vesicles first dock to an early endosome (EE), into which the cargo and receptors are sorted and potentially recycled. As the EEs mature, they grow by undergoing homotypic fusions and start accumulating newly synthesized lysosomal enzymes through the trans-Golgi network (TGN). Ultimately, these late endosomes (LE) fuse with lysosomes to form an endolysosome, a transient structure in which the bulk of the cargo degradation takes place. Reproduced from Huotari and Helenius (2011).

This last step results in the formation of a hybrid organelle, the endolysosome, where the bulk of the degradation occurs through the action of lysosomal enzymes (e.g. cathepsins, phospholipases, glycosidases) (Repnik et al. 2013).

The endosome/lysosome pathway (Figure 2 and above) processes endocytic vesicles resulting from micropinocytosis (Pollard et al. 2017) and some differences exist with the endocytic pathways of phagosome or macropinosomes. Phagosomes are believed to skip the initial pooling and sorting step: they rather conserve their original structure and mature through acidification of the lumen. Maturation occurs by fusing with enzyme-containing Golgi vesicles and ultimately, fusion with a lysosome results in the formation of a phagolysosome where digestion occurs (Kinchen and Ravichandran 2008). The fate

of macropinosomes is less clear, but they are believed to go through a maturation process similar to that of phagosomes (Kerr and Teasdale 2009).

After food particles and/or macromolecules are fully broken down into assimilable nutrient monomers, they exit the lysosome and enter the cytoplasm. They are then either metabolized directly, deposited in storage organelles (e.g. lipid droplets, glycogen granules) or released in the extra-cellular space to be transported towards other tissues (see for example lipid transport, section 3.2) (Martin and Parton 2006; Hartenstein and Martinez 2019). Potential undigestible material accumulates in the lysosome (then termed residual body) (de Duve and Wattiaux 1966; Pollard et al. 2017) and are ultimately excreted out of the cell through exocytosis in most cases (de Duve and Wattiaux 1966).

2.4 Function and evolution of cell types involved in intracellular digestion

Despite a wide diversity of body plans, diets and feeding behaviors, intracellular digestion of food is very similar among animals (Fankboner 2003). Most commonly, cells that take up and digest food are part of the digestive tract epithelium (Figure 3, enteric phagocytes), where they intercalate between gland cells that secrete extracellular digestive enzymes (Hill et al. 2016; Hartenstein and Martinez 2019). The apical membrane of digestive cells, facing toward the lumen of the gut, usually bears specialized structures such as cilia and/or microvilli that help moving food in the gut and increase its absorptive surface, respectively (for a review of the ultrastructure of animal digestive cells, see Hartenstein and Martinez 2019). Exceptions to this organization are found for example in nematodes, which have bi-functional cells capable of both extra- and intra-cellular digestion (Riley 1973). Another striking variation to the epithelial organization of enteric phagocytes occurs in in acoels, where phagocytic cells fuse into a permanent or transient phagocytic syncytium (Figure 3) (Gavilán et al. 2019).

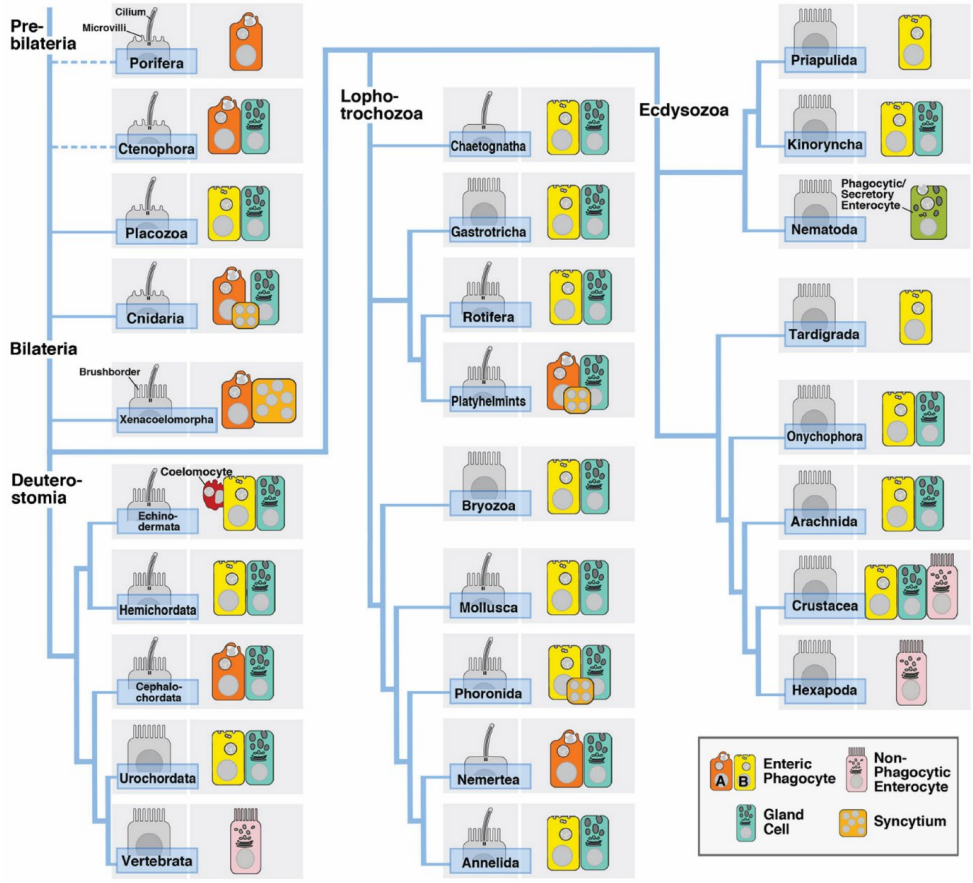

Figure 3: Phylogenetic distribution of digestive cell types in animals. Grey schematic on the left indicates apical membrane structure (e.g. cilia, microvilli). Type A and B of enteric phagocytes correspond to the canonical phagocytosis pathway (as described in section 2.3.1) and to an alternative pathway where smaller particles are taken up in vesicles that subsequently fuse together into a large phagosome (as described in Hartenstein and Martinez 2019). Non-phagocytic enterocytes correspond to absorptive cells that take up single molecules resulting of extracellular digestion. Reproduced from Hartenstein and Martinez (2019).

Enteric phagocytes (also termed ‘trophic phagocytes/endocytes’ thereafter) are present in most invertebrate groups, including in non-bilaterians (Figure 3) (Steinmetz 2019; Hartenstein and Martinez 2019). Sponges, lacking a closed digestive cavity, exclusively perform intracellular digestion via choanocytes, ciliated cells that create a water flow in the organism, internalize and digest food particles (Gonobobleva and Maldonado 2009;

Brusca et al. 2016). Their morphological similarities to choanoflagellates, together with the broad distribution of endocytic cell types among animals, lead some authors to propose that trophic phagocytes might be the most ancient animal cell type (Yonge 1937; Brunet and King 2017; Musser et al. 2019).

It is interesting to note that reviews addressing the intracellular digestion of food in animals focus mostly on the phagocytic activity of digestive cells (Desjardins et al. 2005; Lancaster et al. 2019; Hartenstein and Martinez 2019), although it is known that other endocytic mechanisms also play an important role in this process (McMahon and Boucrot 2011; Pollard et al. 2017). In addition, despite being a crucial physiological process in most invertebrates, intracellular digestion of food has so far been mainly described on the ultrastructural level (reviewed in Hartenstein and Martinez 2019).

Intracellular digestion has additional non-nutritive, immunity-related functions in pathogen clearance (Yutin et al. 2009; Dayel and King 2014). In fact, 'professional' phagocytes of the immune system are among the most extensively described endocytic cell types (Rabinovitch 1995; Gordon 2016; Rosales and Uribe-Querol 2017). In vertebrates, almost exclusively relying on extracellular digestion for nutrition, phagocytosis is a central mechanism during the innate immunity response notably by macrophages (Rabinovitch 1995; Karasov and Hume 2011). Similarly, intracellular digestion has also been described in invertebrate immune cells: clearance of bacteria and exogenous particles in echinoderms and annelids relies on the phagocytic activity of amoeboid coelomocytes, which are motile, chemotactic cells of the coelomic fluid (Johnson 1969; Stein et al. 1977; Gross et al. 1999; Engelmann et al. 2016).

Trophic and immune endocytic cell types display extensive structural and functional similarities. In addition, as some animals feed on bacteria, their roles in 'feeding' and 'immunity' are most often undistinguishable. Illustrating this ambiguity, sponge archaeocytes or echinoderm coelomocytes have been proposed to have a role in both immunity and nutrition (Chia and Koss 1991; Imsiecke 1993; Funayama et al. 2005). Additionally, immune and trophic phagocytes can have similar ontogenic origins: vertebrate immune cells – including phagocytic macrophages – originate in the

mesoderm during development (Herbomel et al. 1999; Fehling et al. 2003; Hartenstein 2006), as do the enteric phagocytes of *C. elegans* (Amin et al. 2010), or the coelomocytes of echinoderms (Smith et al. 2018). Altogether, these observations have fueled hypotheses about a common evolutionary origin of macrophages and enteric phagocytes (Broderick 2015; Hartenstein and Martinez 2019). A deeper understanding of the cellular mechanisms and molecular profiles of trophic endocytes in a comparative approach are necessary to test this assumption.

3. Mobilizing and spending resources: the example of lipid transport during vitellogenesis

After food components have been taken up, digested, and stored in specialized tissues, these nutrients are available for metabolism, growth and reproduction – maintaining energy homeostasis (see section 1) (Hill et al. 2016). Oogenesis is one of the most resource-demanding processes in animals. Oocytes are generally among the largest cells of the body, and the successful early development of an embryo often relies on the nutritive resources accumulated in the eggs during their maturation (Wallace 1985; Gilbert and Barresi 2017). Food availability thus dictates reproduction effort and success, and is a major driver of reproductive seasonality (Eckelbarger 1994; Hahn et al. 2005; Tixier et al. 2015; Alqurashi et al. 2020). Many animals display elaborate mechanisms to regulate oogenesis under scarce food conditions. In *Drosophila*, shifting to a diet of poor nutritional quality induces a decrease in the proliferation of germ-line stem cells and their progeny, leading to a 60-fold decrease in egg production (Drummond-Barbosa and Spradling 2001). Starvation in *C. elegans* was shown to induce germ-cell apoptosis, and this effect was more pronounced than under other types of stress (Salinas et al. 2006). Vitellogenesis, the process during which nutrients such as proteins or lipids accumulate in the growing oocyte, is therefore highly relevant to study the mobilization and use of dietary resources acquired by animals.

3.1 Digestion and absorption of dietary lipids in the bilaterian gut

Lipids are essential building blocks of cell membranes, and are also a major, highly caloric source of energy for metabolism (Wältermann and Steinbüchel 2005; Azeez et

al. 2014). They are stored in the form of cytoplasmic droplets in most cell types, but many animals also possess specialized lipid storage tissues such as the adipose tissue of mammals or the fat body of insects (Walther and Farese 2012; Azeez et al. 2014). Most interestingly, lipids make up a large part of the yolk accumulating in oocytes during vitellogenesis, providing an essential energy source to the embryo during early development (Wiegand 1996; Anton 2007).

In all major bilaterian groups, complex dietary lipids are degraded in the lumen of the gut by intestinal lipases. The resulting products are predominantly free fatty acids and monoglycerides, which are subsequently taken up by the gut epithelial cells (Bauer et al. 2005; Voet et al. 2016). The absorption of lipids by gut enterocytes occurs either by diffusion across the cell membrane or via protein-dependent transport (Iqbal and Hussain 2009; Voet et al. 2016). Notably, the Fatty Acid Translocase/CD36 receptor mediates fatty acid absorption from the lumen of the small intestine in mammals (Nassir et al. 2007), while members of the Fatty Acid Transport Proteins (FATPs) family have a conserved role in lipid uptake from fungi to vertebrates (Hirsch et al. 1998; Stahl et al. 1999). Free fatty acid are quickly incorporated into triacylglycerols (TAGs), which are the main lipid transport or storage form in animals, with palmitic acid and oleic acid being the most abundant fatty acid species (Birsoy et al. 2013; Hill et al. 2016; Voet et al. 2016).

3.2 Lipid transport in bilaterians: the Large Lipid Transfer Proteins family

Due to their hydrophobicity, TAGs and other lipids cannot freely diffuse in the extra-cellular compartment. Lipid transport in animals is thus facilitated by members of the large lipid transfer proteins (LLTP) superfamily, which is highly conserved in metazoans (Babin et al. 1999; Smolenaars et al. 2007). In vertebrates, apolipoproteins (e.g. Apolipoprotein B) associate with dietary lipids to form amphiphilic lipoprotein complexes (Van der Horst et al. 2009; Hussain 2014). In these structures, lipids are packed with different combinations of apolipoproteins, consisting usually of an isoform of Apolipoprotein B (ApoB) and several non-LLTP apolipoproteins (e.g. ApoE) that confer additional properties to the complex (e.g. enhanced receptor-binding) (Innerarity

and Mahley 1978; Voet et al. 2016; Huebbe and Rimbach 2017). Vertebrate lipoproteins have variable sizes and functions. The largest, chylomicrons, are formed in intestinal cells and distribute dietary lipids in the body through the circulatory system. The smaller low density (LDL) and very low density lipoproteins (VLDL) carry endogenous TAGs and cholesterol, mainly from the liver to peripheral tissues via the blood system (Voet et al. 2016). Lipid transport in invertebrates has mostly been examined in insects where Lipophorins are the main lipoproteins, consisting of lipids packed with Apolipophorins (ApoLpp). ApoLpp proteins are also part of the LLTP family and considered orthologous to vertebrate ApoB proteins (Babin et al. 1999; Smolenaars et al. 2007; Palm et al. 2012). Produced in the fat body, Apolipophorins distribute dietary lipids from the gut throughout the body via the hemolymph (Palm et al. 2012). In addition to their role in systemic lipid transport, ApoLpp are also the major proteins delivering lipids to the follicle cells of the ovary during vitellogenesis (Kawooya and Law 1988; Raikhel and Dhadialla 1992). In most other animals, from sponges to vertebrates, lipid transport into developing oocytes is however mediated specifically by Vitellogenin (Vtg), a glycolipoprotein that is also a member of the LLTP family (Wahli et al. 1981; Wallace 1985; Polzonetti-Magni et al. 2004). In insects, Vtg plays only a minor role in vitellogenesis in comparison to apolipophorins (Hayward et al. 2010; Riesgo et al. 2014; Fruttero et al. 2017).

After reaching a target tissue through the circulatory system, lipoproteins are internalized through receptor-mediated endocytosis.

3.3 The Low-density lipoprotein receptor family

The human Low Density Lipoprotein Receptor (LDLR) (Yamamoto et al. 1984) was the first identified and thus name-giving member of the large LDL receptors family. Studying its role in cholesterol metabolism laid the foundations of our understanding of receptor-mediated endocytosis (Brown and Goldstein 1979; Strickland et al. 2002). LDLRs are very conserved in metazoans, and can overall be divided into two groups: (1) endocytic receptors that mediate the uptake of lipoproteins, and (2) receptors involved in signaling pathways (e.g. LRP5/6 in Wnt pathway) (Dieckmann et al. 2010).

Their respective functions are however not fully elucidated. As most of these receptors are able to bind several ligands (e.g. currently over 30 identified ligands for LDLR-Related Protein (LRP)) (Herz and Strickland 2001)), many are multifunctional and can have dual roles in lipoprotein metabolism and signal transduction (Schneider and Nimpf 2003).

Most relevant for this work are 'canonical' LDLR family members with roles the in endocytosis and subsequent lysosomal degradation of lipoproteins. These include vertebrate orthologs of LDLR, LRP1, LRP2, Very Low Density Lipoprotein Receptors (VLDLRs), and the oocyte-specific Vitellogenin receptors which are involved in the cellular uptake of ApoB, ApoE and Vitellogenin (Schonbaum et al. 1995; Schneider 1996; Schneider and Nimpf 2003; Dieckmann et al. 2010). These receptors are expressed in different sets of vertebrate cells and can show specificity for different lipoproteins and lipid contents (Schneider et al. 1999; Strickland et al. 2002; Dieckmann et al. 2010). Vitellogenin receptors, which include orthologs of vertebrate VLDLR and insect Lipophorin receptors, have been shown to be essential for yolk deposition via endocytosis in the eggs of the fly (Schonbaum et al. 1995), chicken (Nimpf and Schneider 1991) and *C. elegans* (Grant and Hirsh 1999).

3.4 Vitellogenesis in bilaterians

The accumulation of nutrients in growing oocytes is a process that varies greatly between species. Eggs can be almost devoid of yolk (alecithal, mainly seen in mammals), contain moderate amounts (oligolecithal, found e.g. in marine animals with planktotrophic larvae) or be very yolk-rich (macrolecithal, found e.g. in birds or cephalopods) (Wourms 1987; Gilbert 2010). The composition of yolk is variable, and usually includes proteins, carbohydrates and lipids in variable proportions (Wourms 1987). In most animals, Vitellogenin is the main yolk precursor, and it is cleaved inside the oocytes into two main products: the lipoprotein Lipovitellin and the phosphoprotein Phosvitin (Wiley and Wallace 1981; Polzonetti-Magni et al. 2004). After being further processed, protein subunits are stored as protein platelets in the cytoplasm while the lipids are stored in lipid droplets (Wourms 1987). In this work, we are mostly interested

in the modalities of vitellogenesis: how are dietary nutrients transported into developing oocytes?

3.4.1 Autosynthetic and heterosynthetic yolk production

As first defined by Schechtman in 1955, there are three different possible modalities of vitellogenesis in animals: (1) autosynthetic, when the yolk is synthesized by the oocyte itself, (2) heterosynthetic when the yolk is produced by somatic cells and subsequently delivered to the oocytes, and (3) a combination of autosynthesis and heterosynthesis.

Autosynthesis has been described most often indirectly by ultrastructural electron microscopy studies, even in most recent publications (e.g. Elbarhoumi et al. 2014; Roy et al. 2020). Yolk produced autosynthetically is believed to be mostly composed of proteins and carbohydrates, and to originate in the endoplasmic reticulum and Golgi apparatus of the oocytes (Anderson 1974). This mode of vitellogenesis is present in almost all animal phyla, and is most often found in combination with heterosynthesis. Examples of autosynthesis in bilaterians include annelids (e.g. Eckelbarger 1980; Pfannenstiel and Grünig 1982; Elbarhoumi et al. 2014), crustaceans (Kessel 1968), mollusks (e.g. Eckelbarger and Young 1997; Kim 2016; Roy et al. 2020), tunicates (e.g. Kessel 1966; Manni et al. 1994), and vertebrates such as amphibians (Kress 1982) and fish (Chung et al. 2009).

Heterosynthesis is similarly most often detected using ultrastructural methods, and is characterized by the presence of endocytic pits and vesicles on the oolemma (e.g. Wallace and Dumont 1968; Eckelbarger 1980). Heterosynthetic vitellogenesis is also widely present among animal groups, but the localization of yolk precursor production and its transport can differ considerably (Eckelbarger 1994). Generally, the yolk can originate either remotely from the ovary, often requiring a long-distance transport to reach the oocytes, or in ovarian accessory cells located near the developing eggs (Wourms 1987; Eckelbarger 1994).

3.4.2 Yolk synthesis in extra-ovarian tissues

Extraovarian synthesis of yolk occurs in cell types located close to or remote from the

ovary, and therefore involves the transport of yolk through the body (Figure 4A). In vertebrates, Vtg is mainly produced in the liver and transported to the ovary via the blood stream (Wallace 1985; Li and Zhang 2017; Reading et al. 2017). Vtg is small enough to penetrate the ovary through the intercellular spaces in-between epithelial cells to reach to the oocytes where it is endocytosed (Neaves 1972; Dumont 1978; Wallace 1985). Similarly, vitellogenin is mainly produced by cells of the hepatopancreas in crustaceans or the fat body in insects (together with lipophorins), and reach the ovary via the hemolymph (Kawooya and Law 1988; Raikhel and Dhadialla 1992; Subramoniam 2011). Interestingly, a defined circulatory system is not a pre-requisite for the occurrence of extraovarian yolk synthesis: in *C. elegans*, yolk proteins are produced by intestinal cells, released in the pseudocoelomic cavity and ultimately taken up by the oocytes (Kimble and Sharrock 1983; Grant and Hirsh 1999).

3.4.3 Yolk synthesis by ovarian accessory cells

In most animals, oocytes maintain a close proximity to specialized cells throughout the maturation process. These so-called ‘accessory cells’ can be part of a complex ovary or form a simple epithelium around the gametes. They usually offer mechanical support and protection to the oocytes, and very often contribute to the production of yolk during vitellogenesis (Adiyodi and Adiyodi 1983; Wourms 1987; Eckelbarger 1994). In contrast to extraovarian yolk, yolk synthesized by accessory cells does not necessitate long distance transport through extracellular compartments. Accessory cells are therefore found even in animals with a very simple body organization such as sponges (Adiyodi and Adiyodi 1983).

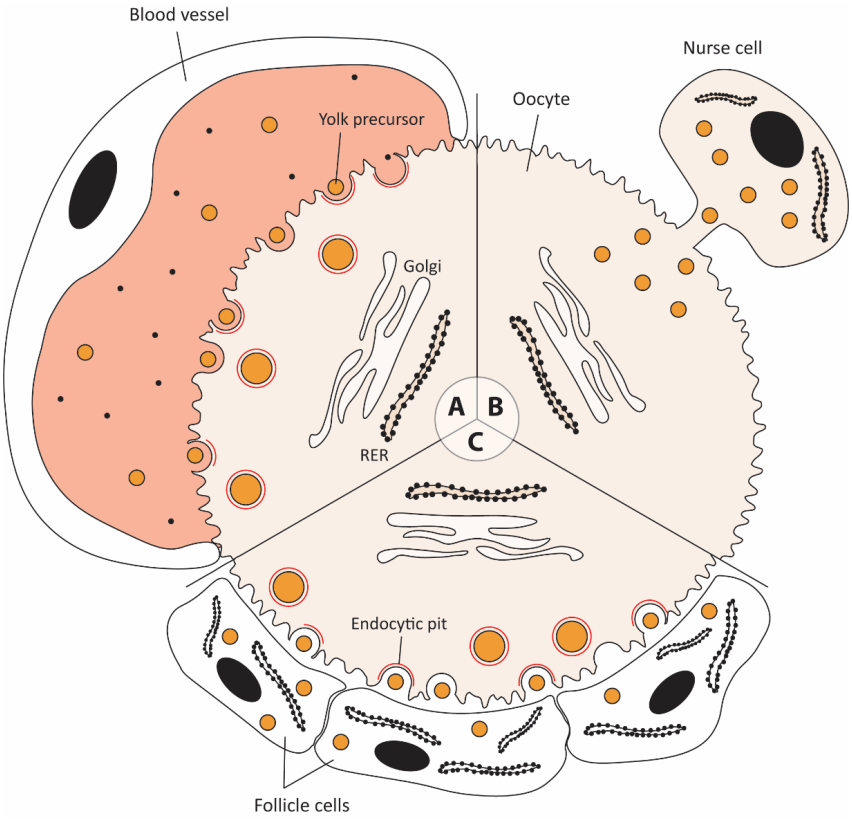

Figure 4: Schematic representation of the three main modalities of heterosynthetic vitellogenesis. (A) Extraovarian-produced yolk (orange circles) is transported in the circulatory system and incorporated in the oocyte (center) via receptor-mediated endocytosis. **(B)** Nurse cell provides yolk through cytoplasmic bridge. **(C)** Follicle cell-produced yolk is endocytosed by the oocyte. Inspired by Eckelbarger 1994.

Follicle cells are the most ubiquitous type of ovarian accessory cells, and are found in most bilaterian groups (Eckelbarger 1994). They are always of somatic origin and are usually organized in a layer surrounding the oocytes (Figure 4C) (Wourms 1987; Eckelbarger 1994). They have been well characterized in insects where yolk lipoproteins are produced by both the fat body and ovarian follicle cells, illustrating the co-occurrence of extraovarian and ovarian yolk production observed in many bilaterians (Telfer et al. 1982; Brennan et al. 1982; Richard et al. 2001). In vertebrates, follicle cells play a critical role in the hormonal regulation of oogenesis (Polzonetti-Magni et al. 2004), but can also contribute to yolk production (Marina et al. 2004).

The second main type of ovarian accessory cells, nurse cells, are commonly defined as abortive germ cells maintaining cytoplasmic bridges with the oocytes (Huebner and Anderson 1976). They provide organelles, nutrients and other maternal factors to the developing eggs through cytoplasmic transfer (Figure 4B), and are in some cases phagocytosed by the developing oocyte (e.g. in *Hydra*)(Adiyodi and Adiyodi 1983). Nurse cells have notably been documented in cnidarians (e.g. *Hydra,* see section 4.3), ctenophores (Hernandez-Nicaise 1991) and in insects where they have been characterized more extensively (reviewed in Telfer 1975; Wourms 1987). Cells presenting morphological and functional similarities with nurse cells have also been described in sponges (Fell 1969), but their classification as 'canonical' nurse cells is difficult. This is due to the fact that sponges do not have a separate germ line, as totipotent archaeocytes are believed to give rise to both oocytes and nurse cells (Adiyodi and Adiyodi 1983; Wourms 1987).

4. Energy homeostasis in non-bilaterian species: insights from cnidarians

Cnidarians include marine and freshwater gelatinous animals that can be pleustonic, pelagic or benthic (often both during the life cycle), free-swimming or sessile, solitary or colonial, and thrive everywhere from the top to the abyssal depths of the water column (Gershwin 2016). They have fascinated biologists with their diversity of sizes and shapes, key roles in ecosystems, elaborate life cycles and regenerations abilities. Moreover, they hold a key position in the metazoan phylogeny as the sister group to bilaterians, making them of great interest for comparative evolutionary studies.

The morphological synapomorphy of cnidarians is a specialized sensory cell type, the cnidocyte, a stinging cell used for predation and defense. Cnidarians are divided into two clades: medusozoans and anthozoans (Figure 5). Anthozoans, the sister group to all other cnidarians, possess a polyp stage but no medusa life stage. They are further sub-divided into (1) hexacorallians, that include sea anemones (e.g. *Nematostella vectensis*, *Exaiptasia diaphana*) and stony corals (e.g. *Acropora millipora)*, and (2) octocorals that group sea pens, soft corals and gorgonians (e.g. *Xenia sp*). Medusozoans regroup all the taxa possessing a medusa stage: hydrozoans (hydroids, hydromedusae, siphonophores; e.g. *Clytia hemispherica*, *Hydra sp*), scyphozoans (true jellyfish, e.g. *Aurelia aurita*),

staurozoans (stalked jellyfish) and cubozoans (box jellyfish, e.g. *Tripedalia cystophora*, *Chironex fleckeri*) (Kayal et al. 2013; Zapata et al. 2015). Some rare species (e.g. *Hydra sp.*) have lost the medusa stage (Technau et al. 2015).

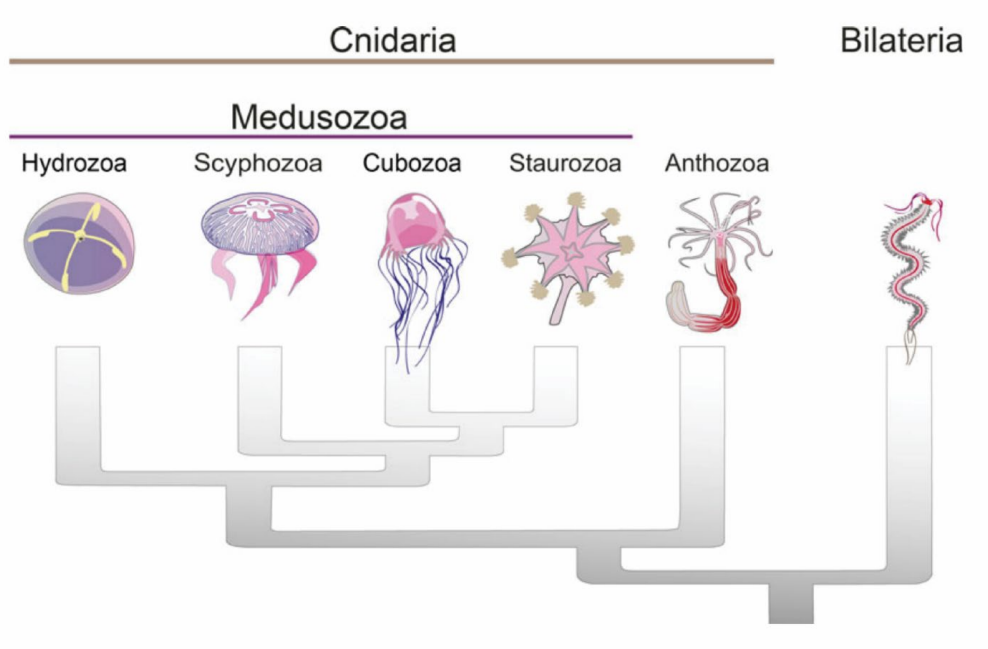

Figure 5: Phylogenetic relationships among major cnidarians groups, based on Collins (2002), Marques and Collins (2005). Anthozoans include sea anemones and represent the outgroup to medusozoans, which includes all other major cnidarians groups. Reproduced from Technau et al. 2015.

The 'simple' body plan of cnidarians is composed of two epithelial cell layers, an inner gastrodermis and an outer epidermis. It possesses a single orifice used for feeding and excretion and lacks a centralized nervous system. Cnidarians display a wide variety of life cycles. In most species, sexual reproduction results in a ciliated planula larva that settles on a substrate, giving rise to a polyp that can reproduce sexually (anthozoans & hydroids) or asexually (medusozoans & anthozoans). In medusozoans, this cycle is completed by a free-swimming medusa stage (Gershwin 2016).

In this work, we use the sea anemone *Nematostella vectensis* as a research organism to

study the evolution of processes linked to energy homeostasis. Considering its strategic phylogenetic position, understanding the physiological and molecular pathways underlying energy homeostasis in *Nematostella* allows us to infer the characteristics of the last common ancestor to cnidarians and bilaterians, thereby shedding light on the evolution of this process among early animals.

4.1 Nematostella vectensis as research organism

4.1.1 Biology

Nematostella vectensis is a small anemone of the family Edwardsiidae, first described by Stephenson in 1935. It is native to shallow pools of brackish water in salt marshes and estuaries on the Atlantic coast of North America, and is also found in similar habitats on the Pacific coast and in England (Sheader et al. 1997; Reitzel et al. 2008). It burrows in the sediment leaving only the tentacles free in the water to catch its prey consisting of small organisms such as copepods, rotifers, insect larvae and mollusks (Hand and Uhlinger 1994). *Nematostella* is dioecious, and sexual reproduction is external (Figure 6).

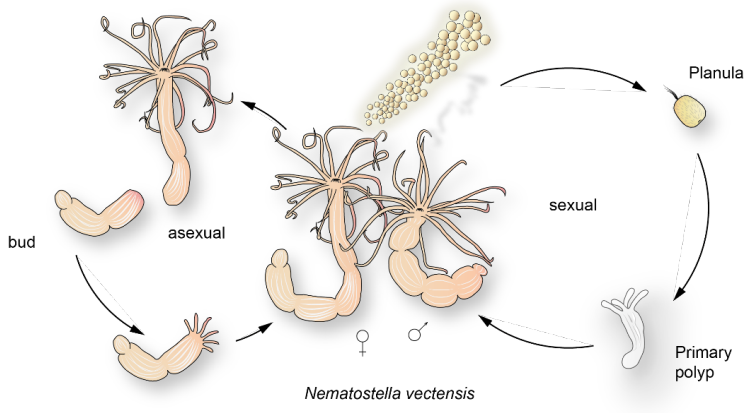

Figure 6: Life cycle of *Nematostella vectensis*, depicting both sexual and asexual reproduction. Mature males and females release gametes in the water, where fertilization occurs. Eggs develop into ciliated planula larvae, that settles on the sediments where they grow into primary polyps and ultimately mature adults. Asexual reproduction occurs through transverse fission:

the extremity of the foot pinches off and regenerate into a mature animal. Artwork by Johanna Kraus.

Females expulse a bundle of eggs embedded in a thick mucus through the mouth, that is fertilized in the water by sperm cells released by the males. Fertilization results in a free-swimming planula larva that settles on the sediment and progressively develops into a mature polyp (Figure 6) (Hand and Uhlinger 1992; Uhlinger 1997; Reitzel et al. 2007). This species is also capable of asexual reproduction: a part of the foot will pinch off and regenerate into a new, identical anemone (Hand and Uhlinger 1995; Reitzel et al. 2007).

4.1.2 Morphology

In its natural habitat, *Nematostella* is usually shorter than 2cm and only a few millimeters in diameter (Hand and Uhlinger 1992). Under laboratory conditions when food is abundant, however, adult polyps can reach sizes larger than 10cm. When disturbed, the animal contracts its body and reduces dramatically in size. The body of the anemone is divided into three regions: the head or capitulum, with a mouth surrounded by tentacles to catch prey, the column (or scapus), and the foot (or physa) that allows burrowing (Figure 7A) (Williams 1975).

As all other cnidarians, *Nematostella* possesses two cell layers: an outer epidermis and an inner gastrodermis separated by a layer of extracellular matrix, the mesoglea (Frank and Bleakney 1976; Martindale et al. 2004). Along the oral-aboral axis, the gastrodermis of the body column exhibits eight inner folds, the mesenteries (Figure 7A-B), which play central roles in digestion, reproduction and food storage (Shick 1991; Steinmetz et al. 2017). Their general morphology changes along the oral-aboral axis but has not been formally named previously. In this manuscript, we have therefore named the oral, gonadal part of the mesenteries ‘gonad region’ and the non-reproductive, aboral part ‘trophic region’ (Figure 7B). The distal tip of the mesenteries – commonly called septal filament – presents a characteristic trilobed organization in parts of the gonad region (Figure 7C). It is subdivided into four distinct structures: (1) the cnidoglandular tract (CGT), bearing numerous cnidocytes and gland cells secreting enzymes for extracellular

digestion (Steinmetz et al. 2017), (2) the intermediate tract (IT), a highly endocytic structure (Van-Praët 1978, 1980; see also paper I), (3) the ciliated tract (CT) that creates a water flow in the gastric cavity (Van-Praët 1985) and (4) the reticulate tract (RT) (showing 'reticulation' patterns in histological cross-sections) that also shows some phagocytic activity (Van-Praët 1978) and harbors potential germline stem cells (Miramón-Puértolas P., unpublished). More proximally, gametes develop in the mesoglea in-between the mesenterial epithelial layers (Figure 7C).

In the trophic region (and parts of the gonadal region, not shown), the septal filament is 'unilobed', i.e. only composed of the cnidoglandular tract (Figure 7D). The middle part of the mesentery consists of the endocytic trophic tract (see Paper 1) but is devoid of gametes. This structure is not well characterized (see Papers 1 and 2) and a region of similar morphology in juvenile polyps has previously been shown to participate in nutrient storage (Steinmetz et al. 2017). Finally, the longitudinal retractor and parietal muscles occupy the basal parts of the mesentery close to the body wall and allow contractions along the oral-aboral axis (Figure 7C-D).

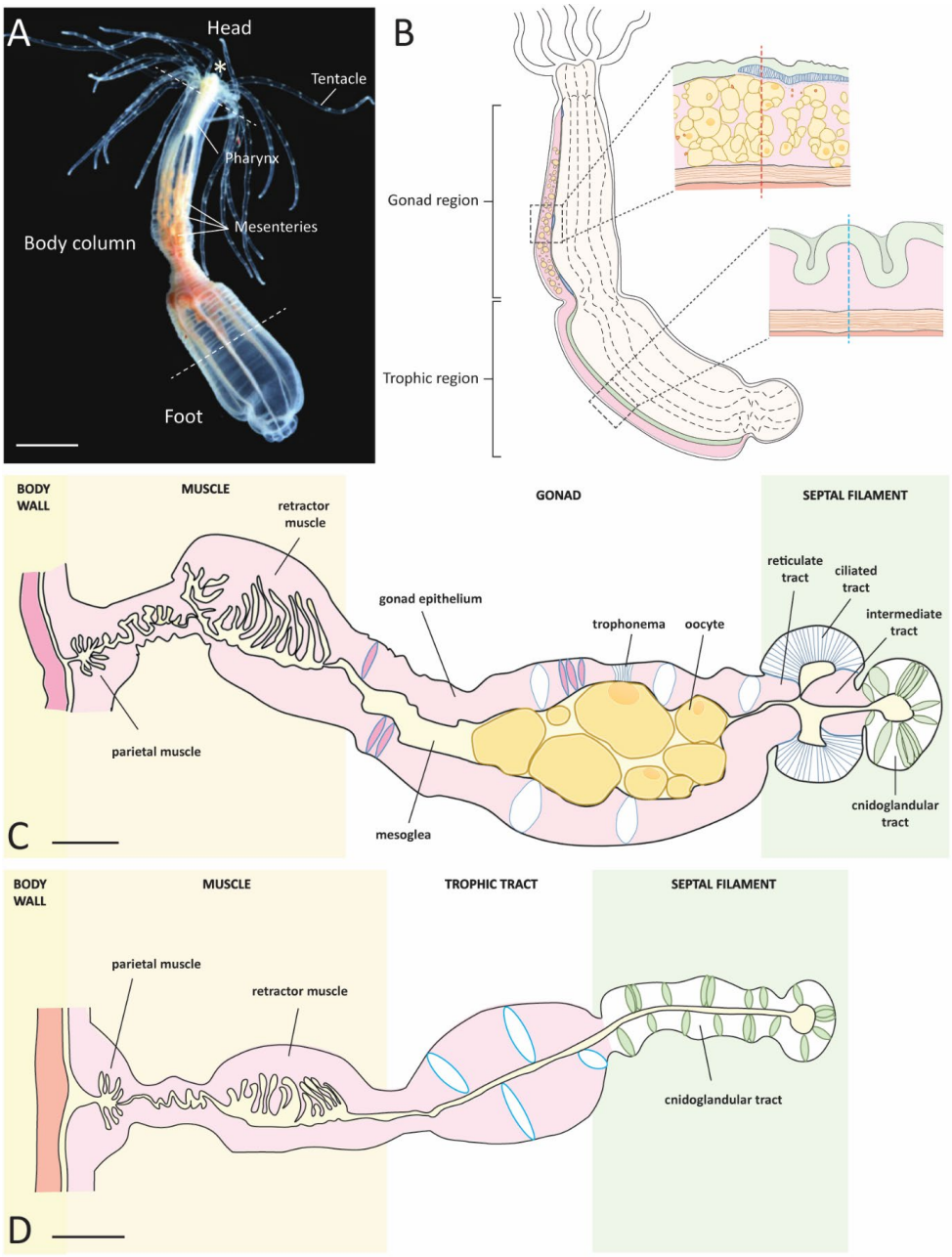

Figure 7: Morphology of *Nematostella vectensis*. (A) Picture of an adult polyp showing the three main subdivisions of the body. Mouth marked with an asterisk. **(B)** Schematic representation of an adult polyp showing the general organization of a mesentery, with a close-up of the gonad and trophic region. **(C)** Schematic representation of a cross section through the

gonad region of an adult female mesentery (marked with a dotted red line in **B**). Males have a similar morphology to females except for spermaries developing in place of oocytes in the gonad region. **(D)** Schematic representation of a cross-section through the trophic region of an adult mesentery (marked with a dotted blue line in **A**). Scale bars: **(A)** 1cm **(C-D)** 50µm. Note that the size and proportion differences between **(C)** and **(D)** reflect reality. **(A)** Adapted from Layden et al. 2016. Gonad region artwork in **(B)** and **(C)** by Paula Miramón-Puértolas.

4.1.3 Nematostella vectensis as an evo-devo research organism

Nematostella has been an increasingly popular cnidarian research organism for evolutionary and developmental studies in the past 20 years (Reitzel et al. 2007; Layden et al. 2016; Steinmetz et al. 2017). It can adapt to a large array of environmental conditions, and is easily kept in the laboratory where it readily reproduces both sexually and asexually. Spawning in males and females can be reliably induced with simple light and temperature variations, providing a steady supply of animals at all stages of development with a relatively short (3-6 months) generation time. A genome assembly of *Nematostella* is available since 2007 (Putnam et al. 2007), and was recently improved to chromosome-level quality using the latest methodologies (Zimmermann et al. 2020). It has facilitated the development and implementation of a wide array of molecular techniques such as *in situ* hybridization (Genikhovich and Technau 2009), transgenesis (Renfer et al. 2010; Renfer and Technau 2017), morpholino-mediated gene knock-down (Magie et al. 2007; Rentzsch et al. 2008) or CRISPR/Cas9 genome editing (Ikmi et al. 2014).

Particularly relevant for the present study, oogenesis and food uptake are tightly linked in *Nematostella*. A long-term experiment revealed that starvation leads to a slow decrease in egg production (Figure 8), confirming the suitability of *Nematostella* for the study of food uptake and vitellogenesis in the context of energy homeostasis.

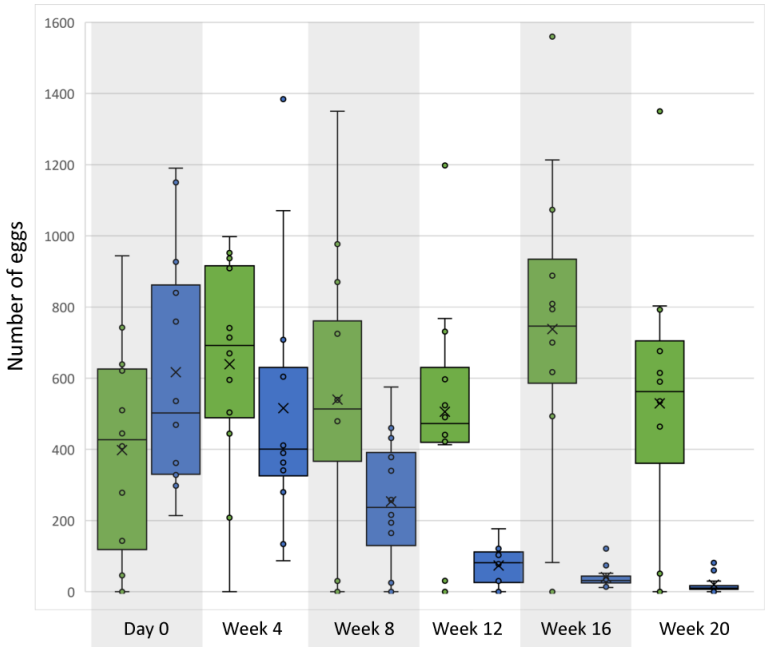

Figure 8: Decrease in egg production over a long-term starvation period in female *Nematostella*. Adult female polyps fed normally (green) or fasted (blue) were induced for spawning every two weeks. The eggs were collected, photographed and counted using an imaging software (Fiji). Egg production slowly decreased over a 20-weeks period in starved animals, while egg production in fed females remained high. n = 12 in both groups.

4.2 Extra- and intracellular digestion in cnidarians

In general, cnidarians are carnivorous predators that capture and incapacitate their preys using cnidocyte-covered tentacles. Preys are subsequently inserted into the gastrovascular cavity through the mouth (Brusca et al. 2016). Digestion in cnidarians consists first of an extracellular digestion in the gastrovascular cavity (GVC), then in the uptake of resulting food particles into gastrodermal cells where digestion is finalized (Fautin and Mariscal 1991; Lesh-Laurie 1991; Thomas and Edwards 1991). Extracellular digestion and particle uptake is often enhanced by water currents created by ciliary flow in the GVC. In medusae, food particles are distributed throughout the body through radial canals that originate in the stomach and connect to the bell rim (Brusca et al. 2016). The morphology of the GVC is variable between the four main cnidarian groups (Figure 9), resulting in differences in the anatomy of digestion

(reviewed in Steinmetz 2019). Notably, the regions and cell types involved in endocytosis are still poorly described or unknown in most cnidarian groups (e.g. cubozoans, scyphozoans or staurozoans).

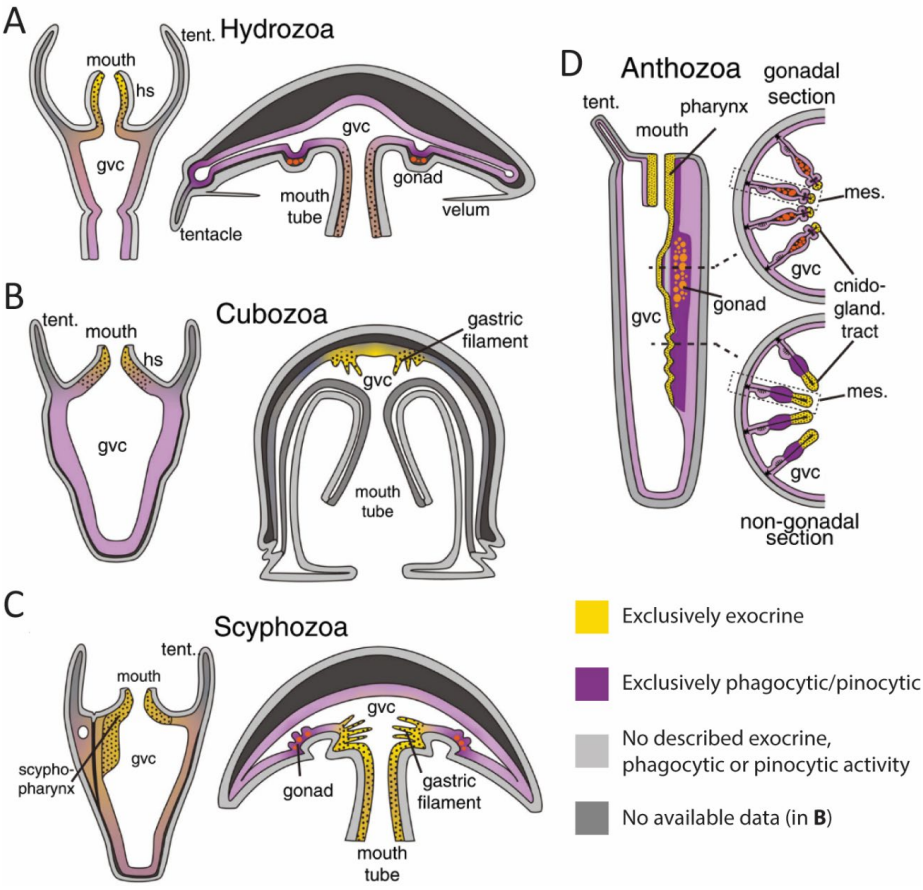

Figure 9: Schematic representation of the GVC in hydrozoans (A), cubozoans (B), scyphozoans (C) and anthozoans (D) with a focus on the distribution of tissues involved in extracellular and intracellular digestion. gvc: gastrovascular cavity; hs: hypostome; mes: mesentery; tent: tentacle. Adapted from Steinmetz 2019.

Hydrozoan polyps have a simple sack-like GVC with no compartmentalization or specialized structures (Figure 9A) (Thomas and Edwards 1991; Bouillon et al. 2006). Digestive, exocrine gland cells generally locate to the polyp mouth region, while phagocytic and endocytic activities occur in the body column (Thomas and Edwards 1991; Bouillon et al. 2006). Interestingly, in the well-described hydrozoan *Hydra vulgaris,* digestive gland cells are not restricted to oral regions, but unusually intercalate among endocytic cells along the entire body column (Haynes and Davis 1969). In hydrozoan medusae, digestive enzyme secretion similarly occurs by gland cells in the mouth tube while endocytic cells line the gastric cavity (Bouillon et al. 2006). As in anthozoans and scyphozoans (see below & paper 1), the gastrodermis surrounding the gametes seems to be a region of increased endocytic activity (e.g. *Clytia hemisphaerica*) (Roosen-Runge 1962; Amiel et al. 2010)

The inner structure and distribution of digestive regions (both extra- and intracellular) of cubozoan polyps is similar to hydrozoan polyps (Figure 9B) (Chapman 1978; Laska-Mehnert 1985). Cubomedusae present specialized digestive structures located aborally in the GVC, the gastric cirri (Figure 9B), which contain cnidocytes and digestive enzyme-secreting gland cells and are thus similar to the cnidoglandular tract of sea anemones (Di Camillo et al. 2006; Bentlage and Lewis 2012). A recent transcriptomic analysis in *Alatina alata* confirmed the expression of both toxin genes and digestive enzyme genes (e.g. *chymotrypsin-like* genes) in these structures (Lewis Ames et al. 2016). To my knowledge, intracellular digestion in cubomedusae has not been studied yet.

In contrast to hydrozoan and cubozoan polyps, scyphozoan polyps are compartmentalized: the gastrodermis extends four septa into the GVC, delimitating four interconnected gastric compartments (Lesh-Laurie 1991). Digestive gland cells are concentrated in the septa and endocytic activity has been described throughout the gastrodermis (Figure 9C) (Lesh-Laurie 1991; Arai 1996). Scyphomedusae have been studied more extensively than the polyp stage, particularly in *Aurelia aurita*. In *Aurelia* medusae, *in situ* hybridization studies showed the expression of extracellular digestive enzymes (e.g. *trypsins*, *chitinases*, *pancreatic lipases*) in gland cells located along the

gastric cirri and mouth tube (Steinmetz et al. 2017). Endocytic activity is present throughout the gastrodermis and is particularly intense in the somatic gonad area (Lesh-Laurie 1991; Arai 1996; Steinmetz 2019).

Anthozoans display the most complex polyp organization among cnidarians. As described for *Nematostella*, the gastrodermis of the GVC folds into bilateral-symmetrical pairs of mesenteries (see 4.1.2) (Fautin and Mariscal 1991). The cnidoglandular tract (see Figure 7C-D) at the tip of the mesenteries is composed of cnidocytes and gland cells, and is therefore functionally similar to the gastric cirri of scypho- and cubomedusae (Krijgsman and Talbot 1953; Van-Praët 1985; Fautin and Mariscal 1991). Gland cells of the cnidoglandular tract have been shown to express *trypsins*, *chitinases* and *pancreatic lipases* in *Nematostella* (Steinmetz et al. 2017) and *chymotrypsinogens* in the coral *Stylophora pistillata* (Raz-Bahat et al. 2017). Endocytosis of food particles occurs throughout the gastrodermis with the exception of the cnidoglandular tract and ciliated tract of the mesenteries (Figure 7C-D) (Van-Praët 1985; Shick 1991; Bumann 1995). An increased endocytic activity has been described in the intermediate tract of the sea anemone *Actinia equina* (Van-Praët 1980).

In general, while extracellular enzymatic activity has been described in most cnidarian phyla, studies of intracellular digestion beyond the morphological and micro-anatomical level are rare in the literature. The expression of lysosomal enzymes in cnidarian tissues has been detected only recently through bulk or single-cell transcriptomic analyses, as for example in *Nematostella* (Sebé-Pedrós et al. 2018) and *Aiptasia pallida* (Ishii et al. 2019), or in the hydromedusa *Clytia hemisphaerica* (Chari et al. 2021). More work is necessary to elucidate the intracellular phase of digestion in cnidarians, and this thesis provides first insights into the physiology and molecular machinery underlying this process in *Nematostella*.

4.3 Vitellogenesis in cnidarians

Gametogenesis in medusae occurs in gonadal structures located on the manubrium, the radial canals or the surface of the GVC (Lesh-Laurie 1991; Arai 1996; Bouillon et al. 2006). In anthozoans, gametes develop in the middle part of the mesenteries (Figure

7C), while in hydrozoan polyps, they develop from discrete germ cell patches in the epidermis of the body column (Honegger et al. 1989; Bouillon et al. 2006). For the purpose of this work, I will focus here on female gametes. All cnidarian eggs are lecithal, and several modalities of vitellogenesis have been described in this group. Yolk production occurs to some extent autosynthetically in most species (Anderson 1974; Wourms 1987; Eckelbarger 1994), but ultrastructural and few molecular studies support also heterosynthesis in hydrozoans, scyphozoans and anthozoans.

In *Hydra*, egg patches in the body column epidermis contain large numbers of germ cells of which one becomes a mature oocyte while the rest differentiates into nurse cells (Honegger et al. 1989; Miller et al. 2000). Nurse cells transfer cytoplasmic material to the oocyte through cytoplasmic bridges until they eventually become apoptotic and are phagocytosed by the developing egg (Technau et al. 2003; Alexandrova et al. 2005). Nurse cells have also been observed in some hydromedusae, but are notably lacking in the hydrozoan model *Clytia* where vitellogenesis has not been further described (Amiel et al. 2010).

In cubozoans, the structure of the female gonad shows oocytes developing freely in the mesoglea without contacting the gastrodermis, as observed in *Tripedalia cystophora* (Helmark and Garm 2019), *Copula sivickisi* (Garm et al. 2015) and *Carybdea branchi* (Mohamed et al. 2019). Although vitellogenesis has not been studied specifically, these observations suggest that yolk is transported to the oocytes through the somatic cells and/or mesoglea of the gonad.

In most scyphozoans, oocytes originate in the gastrodermis and bulge into the mesoglea where they undergo vitellogenesis. They maintain a close contact with specialized somatic cells, the trophocytes (Eckelbarger and Larson 1988, 1992; Arai 1996; Ikeda et al. 2011; Schiariti et al. 2012). These cells do not fit the definition of nurse cells as they do not exchange cytoplasm with the oocytes, but have nonetheless been attributed a nutritive role. This hypothesis is based on ultrastructural observations of endocytic activity in trophocytes and the underlying oolemma, and on the presence of yolk granules in the vicinity of the trophocytes (Eckelbarger and Larson 1988, 1992; Ikeda

et al. 2011). However, an early autoradiographic study conducted in *Pelagia noctiluca* failed to demonstrate a transfer of yolk precursors from the trophocytes to the egg (Avian et al. 1987). In *Aurelia aurita*, endocytic pits and vesicles were observed over the whole surface of the oocytes, suggesting that the uptake of yolk is not restricted to the region of the ECM adjacent to trophocytes (Eckelbarger and Larson 1988). Coronate scyphozoans (e.g. *Linuche unguilata*, *Periphylla periphylla*) appear to lack trophocytes, and the oocytes mature freely in the mesoglea (Eckelbarger and Larson 1992; Morandini and Silveira 2001; Lucas and Reed 2009). Oocytes of *L. unguilata* and *Stomopholus meleagris* display endocytic pits on the oolemma, suggesting again endocytosis of yolk from the mesoglea (Eckelbarger and Larson 1992).

Accessory cells similar to scyphozoan trophocytes are also present in anthozoans, where they form a specialized structure called trophonema (Fautin and Mariscal 1991; Shick 1991). Trophonemata were so far described in all anthozoan groups with the exception of sea pens and scleractinian corals (Eckelbarger et al. 2008 and citations within; Shikina and Chang 2016; Lauretta et al. 2018), and the ultrastructure of these cells was first characterized in *Nematostella* (Figure 10) (Eckelbarger et al. 2008). Similar to trophocytes, the localization of trophonemata at the interface between the GVC and the oocytes prompted the hypothesis of a nutritive role. This was partially supported by a study in the sea anemone *Actinia fragacea* where the uptake of radiolabeled amino acids and glucose appeared to be increased in the trophonema cells compared to the rest of the somatic gonad (Larkman and Carter 1982). In addition, an endocytic activity over the entire oolemma was reported in *Nematostella* (Eckelbarger et al. 2008) and *Actinia fragacea* (Larkman 1983). Follicle cells have also been described in the sea pen *Pennatula aculeata*, where oocyte maturation is completed in the GVC (Eckelbarger et al. 1998).

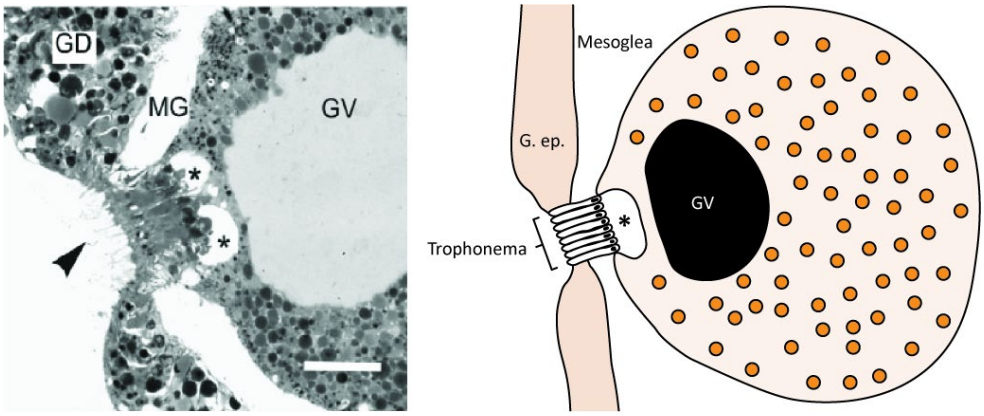

Figure 10: Ultrastructure and schematic representation of the trophonema of *Nematostella vectensis*. Each maturing oocyte contacts the gonad epithelium through a group of columnar cells collectively termed trophonema. Adapted from Eckelbarger et al. 2008. GD: gastrodermis, G. ep.: gonad epithelium, GV: Germinal vesicle, MG: mesoglea, Troph.: trophonema. Asterisk indicates the paratrophonemal space, of unknown function.

Overall, the information introduced thus far demonstrate the high diversity of heterosynthetic vitellogenesis modalities in cnidarians based on morphological studies. More recent studies have shed some light on the molecular mechanisms underlying this process. As in bilaterians, Vitellogenin appears to play a central role during vitellogenesis in cnidarians: the first non-bilaterian *vitellogenin* gene was cloned in the scleractinian coral *Galaxea fascicularis* (Hayakawa et al. 2006). A proteomic study showed that Vitellogenin is the most abundant protein in the mature eggs of *Nematostella* (Lotan et al. 2014). Remarkably, the *vitellogenin* gene in this species appears to be expressed predominantly in the somatic gonad, confirming a major role for this region during vitellogenesis (Levitan et al. 2015). In the coral *Euphyllia ancora*, the RNA expression of *vitellogenin* as well as two other egg yolk components, *egg protein* and *euphy*, were similarly detected in the female gonad epithelium (Shikina et al. 2013, 2015). The corresponding proteins were found in oocytes at different stages of maturation (Shikina et al. 2013). In *Acropora tenuis*, Vitellogenin protein is found in the gonad epithelium and oocytes (Tan et al. 2020). A transcriptomic study in the cubozoan *Alatina alata* revealed the presence of a *vitellogenin* ortholog in a medusozoan (Lewis Ames et al. 2016), but no other orthologs have been published from any scyphozoan or

hydrozoan. Overall, the molecular characterization of vitellogenesis in cnidarians is poor, and the dynamics of this process are unknown.

5. Aims of the study

The present thesis aims to help understanding how cells and physiological pathways underlying energy homeostasis have evolved in animals. Using the sea anemone *Nematostella vectensis* as a cnidarian research organism, we focus on two different aspects of the energy balance: digestion and vitellogenesis. The different parts of this project all contribute to reconstructing the path of nutrients from their digestion to their incorporation in developing oocytes in *Nematostella.* We thereby aim to shed light on the physiological, cellular and molecular processes underlying energy acquisition and consumption in a non-bilaterian species.

Food uptake and intracellular digestion occur via conserved endocytic pathways in specialized regions of the gastrodermis in *Nematostella vectensis* (Paper 1)

Digestion of food in bilaterians most often occurs extracellularly in the gut lumen, into which digestive enzymes are secreted. In bilaterian animals possessing less complex digestive systems as well as in non-bilaterian species, digestion also occurs intracellularly: food particles are internalized into digestive cells by endocytosis and degraded in lysosomes. Intracellular digestion is believed to be the ancestral mode of food digestion in animals. The vast majority of studies, however, has studied this mechanism solely in the context of innate immunity in genetic model organisms (e.g. vertebrate macrophages digesting pathogenic bacteria). The main goal of my first project is therefore to get an understanding of the physiological and molecular pathways at play during the intracellular digestion of food in a sea anemone. This data forms the basis to start comparing trophic endocytic cells in *Nematostella* with phagocytic immune cells of bilaterians: given that these cell types ultimately perform very similar tasks, how do they relate from an evolutionary point of view?

Conserved lipoprotein-LDL receptor pair potentially mediates lipid transport during vitellogenesis in the sea anemone *Nematostella vectensis* (Paper 2)

Vitellogenesis involves the transport of lipids either from extraovarian locations or from ovarian cells. Modalities of vitellogenesis are distributed independently of the presence of a developed circulatory system, making it a fascinating process to study from an evolutionary perspective and in the more general context of lipid transport in animals. As vitellogenesis is poorly characterized in cnidarians, the sister group to bilaterians, my second project aims at investigating the dynamics and molecular machinery at play during lipid transport into developing oocytes in *Nematostella*. By investigating this process, I also seek to uncover potential systemic lipid transport mechanisms in a non-bilaterian, which might help understanding the evolution of animal circulatory systems.

Single-cell atlas of the *Nematostella vectensis* mesentery uncovers a diversity of digestive cells and their transcriptional profiles (Paper 3)

Mesenteries, the gastrodermal folds that line the gastric cavity of anthozoans, are essential for both reproduction and digestion – as illustrated in Papers 1 and 2. Physiological assays and candidate gene *in situ* hybridizations are useful approaches to understand the function of these structures, but they present limitations as they do not allow for an in-depth molecular characterization of cell types. We therefore performed single-cell RNA-sequencing (scRNA-seq) on the mesenteries of adult female polyps, with the goal of better characterizing the diversity and transcriptomic repertoires of the cell types present in mature *Nematostella* mesenteries. These include cells involved in extra- and intracellular digestion, nutrient uptake, reproduction, and other crucial cell types such as stem cells and neurosensory cells. In Paper 3, we present the results of a preliminary analysis of our scRNA-seq dataset focusing on the molecular characterization of cells involved in digestion and food uptake.

Chapter 2: Summary of the results

1. Food uptake and intracellular digestion occur via conserved endocytic pathways in specialized regions of the gastrodermis in *Nematostella vectensis* (Paper 1)

Despite being generally regarded as the ancestral food uptake and processing modality in animals (Yutin et al. 2009; Dayel and King 2014), endocytosis and intracellular digestion have seldom been studied in the context of nutrition. In Paper 1, using physiological and molecular assays, we investigated the uptake of food particles via endocytic mechanisms and their subsequent intracellular digestion in *Nematostella vectensis*.

A first particle uptake assay using large (1µm) fluorescent latex beads (fluospheres) revealed that several regions of the adult mesentery display high phagocytic activity: fluospheres were taken up by cells of the intermediate tract, gonad epithelium and trophic tract **(Paper 1, Figure 1A, B)**. Using a range of incubation times, we were able to show that phagocytosis in *Nematostella* starts within the first 30 minutes after bead ingestion **(Paper 1, Figure 1C-C'')**. While all cells of the intermediate tract appear to take up latex beads, only a subset of cells in the gonad epithelium and trophic tract appear to be phagocytic **(Paper 1, Figure 1D-E'')**. Similar results were obtained when using fluorescent *E. coli* in place of latex beads, suggesting that fluospheres trigger a physiologically relevant uptake response in *Nematostella* **(Paper 1, Figure 1F-F'')**.

Phagocytosis as a mechanism for large particle uptake was further supported by *in situ* hybridization (ISH) data. We observed the specific expression of bacterial-membrane recognition genes involved in phagocytosis in bilaterians (*lipopolysaccharide binding protein/bactericidal permeability increasing protein* (*LBP/BPI*) and *mannose receptor*) **(Paper 1, Figure 2A-B'')** in the intermediate tract, gonad epithelium and trophic tract, and similar expression patterns for three actin-remodeling genes (*cdc42*, *elmo* and *rhoA*) that are core regulators of phagocytosis **(Paper 1, Figure 2C-E'')**.

We further investigated whether mesenteries in *Nematostella* take up smaller particles,

which would be indicative of other endocytic mechanisms. We therefore performed a particle uptake assay using 20nm fluospheres, which were interestingly taken up in the same regions of the mesenteries as their larger counterparts **(Paper 1, Figure 3A, B)**. This result suggests the co-occurrence of several particle uptake mechanisms in these structures and enabled us to demonstrate via ISH that indeed, genes typically linked to receptor-mediated endocytosis were expressed in the endocytic regions of the mesentery. The intermediate tract, gonad epithelium and trophic tract all expressed *low density lipoprotein receptor 4* (*Nv-ldlr4*), an endocytosis receptor of the LDLR family, as well as two clathrin genes (*clathrin light* and *heavy chain*) **(Paper 1, Figure 4)**.

Additional pulse-chase experiments over 14 days using 20nm fluospheres were performed to study the fate of the ingested fluospheres in the polyps in the longer term. We observed that the beads were overall retained in the cells, although some may have been excreted from the trophic tract within the experimental time frame **(Paper 1, Figure 3C-E'')**. There was no evidence of nano-particle transport between tissues. A bi-weekly exposure to 20nm beads over a period of a month resulted in a strong accumulation of fluospheres in vesicles of various sizes located mainly in the apical region of endocytic cells **(Paper 1, Figure 3F, G)**.

After food particles are taken up by endocytic cells, they are digested by intracellular digestive enzymes in the endosome/lysosome system. Using ISH, we found strong and specific expression of genes encoding for lysosomal enzymes (*α-glucosidase*, *α-mannosidase*, *cathepsins*) **(Paper 1, Figure 5 B-E'')** or lysosome-endosome fusion regulator proteins (Rab GTPase *rab2*) in endocytic regions of the mesentery **(Paper 1, Figure 5A-A'')**. This confirms the central role of the intermediate tract, gonad epithelium and trophic tract in the uptake and intra-cellular digestion of food in *Nematostella*.

Finally, we studied the expression of the bilaterian mesodermal marker genes *foxC* and *six4/5* in *Nematostella*, in an effort to explore the molecular relationship between cnidarian endocytic cells and bilaterian mesoderm-derived innate immune phagocytes, the most extensively studied endocytic cells in animals. We found these two

transcription factors to be expressed in the endocytic regions of the mesentery, a result that aligns with previous hypotheses that suggest a common evolutionary origin for the trophic and immune phagocytes **(Paper 1, Figure 6)**. This observation additionally suggests that the endocytic regions of the mesenteries may share a common ontogenic origin with the trophic tract of juvenile polyps, a structure characterized by the expression of *foxC* and *six4/5* and that plays a role in nutrient uptake and storage (Steinmetz et al. 2017).

2. Conserved lipoprotein-LDL receptor pair potentially mediates lipid transport during vitellogenesis in the sea anemone *Nematostella vectensis* (Paper 2)

Vitellogenesis, the accumulation of nutrients in growing oocytes, involves an extensive transport of lipids in most animals including *Nematostella* **(Paper 2, Figure S1)** (Polzonetti-Magni et al. 2004; Ziegler and Van Antwerpen 2006; Voet et al. 2016). We therefore decided to study this process as a model for understanding the fate of dietary lipids in the context of energy homeostasis in sea anemones, and thereby the evolution of systemic lipid transport in animals.

We first assessed lipid uptake in adult female mesenteries by feeding *Nematostella* with *Artemia* nauplii enriched with alkyne-oleic acid (alkyne-OA), a modified fatty acid that can be visualized using 'Click chemistry'. A 20 hours or 7 days alkyne-OA pulse-chase experiment revealed oleic acid uptake in the trophic tract, intermediate tract, gonad epithelium and developing oocytes **(Paper 2, Figure 1)**. We observed a shift of the signal from the distal-most region towards the center of the trophic tract between 20 hours and 7 days of chase **(Paper 2, Figure 1B, C)**. Strikingly, alkyne-OA-positive vesicles were observed in the extracellular matrix (mesoglea) of the trophic tract after 7 days **(Paper 2, Figure 1E)**. This suggests a potential lipid transport from the aboral part of the mesentery towards other regions of the polyp body. In the intermediate tract, alkyne-OA signal was observed after 20 hours of chase but not after 7 days, suggesting that a depletion of alkyne-OA either through intra-cellular metabolism or extra-cellular transport **(Paper 2, Figure 1F, G)**. The incorporation of alkyne-OA into growing oocytes indicated that the compound was a suitable marker to study fatty acid transport

during vitellogenesis in *Nematostella* **(Paper 2, Figure 1H, I)**.

Using short alkyne-OA incubation periods, we observed fatty acid uptake at the apical end of gonad epithelial cells within the first 30 minutes after ingestion **(Paper 2, Figure 2A)**. After 1 hour of exposure, additional small alkyne-OA-positive vesicles became visible towards the base of the epithelium **(Paper 2, Figure 2B)** and within 2 hours of incubation, alkyne-OA was detected in the cytoplasm of the oocytes **(Paper 2, Figure 2C)**. Signal in the developing eggs appeared to be specifically enriched in the oocyte regions adjacent to the gonad epithelium **(Paper 2, Figure 2D)**. Altogether, our results strongly suggest a fast (< 2h) trans-epithelial transport of lipids during vitellogenesis in *Nematostella*. Notably, we did not observe any alkyne-OA uptake in the trophonema, a specialized epithelial structure linking the oocytes to the somatic gonad and historically believed to play a crucial role in nutrient transport during anthozoan vitellogenesis **(Paper 2, Figure 2E)**.

Confirming previously published data (Hayward et al. 2010; Levitan et al. 2015), we found an ortholog of the conserved yolk precursor gene *vitellogenin* expressed exclusively in the gonad epithelium **(Paper 2, Figure 3A-A'', S4)**. A *Nematostella* ortholog of the *apoB* gene, involved in bilaterian systemic lipid transport, was found to be expressed in the gonad epithelium, intermediate tract and trophic tract regions **(Paper 2, Figure 3B-B'', Figure S5)**. In addition to its expression in the gonad epithelium, ApoB is closely related to Vitellogenin **(Paper 2, Figure S4)** and presents a similar domain structure (Hayward et al. 2010), indicating that it may also contribute to lipid transport into the oocytes. Its broader expression pattern in the mesentery also suggests a potential role in lipid transport beyond vitellogenesis.

To test these hypotheses, we used CRISPR-Cas9 to generate a genomic knock-in line expressing a fluorescent ApoB-PSmOrange fusion protein **(Paper 2, Figure 4)**. In female polyps, we found the ApoB fusion protein localized in regions broadly overlapping with RNA expression patterns **(Paper 2, Figure 4A-A'')**. We surprisingly could not detect ApoB-PSmOrange in growing oocytes using this assay, suggesting that ApoB is not internalized to a significant extent during lipid transport into the eggs and

is therefore unlikely to play a major role during vitellogenesis. Surprisingly, however, ApoB may be involved in spermatogenesis: in the male gonad region, the fusion protein was detected in small vesicles associated with developing sperm cells in the spermaries **(Paper 2, Figure 4B-B'')**. ApoB-PSmOrange was also prominent in the trophic tract **(Paper 2, Figure 4C-C'')**, further reinforcing our hypothesis of lipid transport occurring from or within this structure.

Finally, we decided to investigate the molecular machinery underlying lipid uptake during vitellogenesis in *Nematostella*. We identified several orthologs of receptors belonging to the low-density lipoprotein receptor (LDLR) family **(Paper 2, Figures S6, S7)** and could show that three of these genes (*vldlrA*, *vldlrB*, *lrp1*) are specifically expressed at different stages of oocyte growth **(Paper 2, Figure 3C-E'')**. We therefor reveal the presence of a conserved lipoprotein-LDL receptor pair in *Nematostella*, indicating that the molecular mechanisms underlying vitellogenesis in this species are very reminiscent of bilaterian systemic lipid transport despite the absence of a circulatory system.

3. Single-cell atlas of the *Nematostella vectensis* mesentery uncovers a diversity of digestive cells and their transcriptional profiles (Paper 3)

Uncovering the diversity of cell types and transcriptional repertoires present in the mesenteries is essential to better characterize these structures that play key roles in digestion and reproduction in anthozoans. Building upon a previously published single-cell RNA-sequencing (scRNA-seq) study conducted on sexually immature polyps (Sebé-Pedrós et al. 2018), we performed targeted scRNA-seq on the microdissected mesenteries of adult female *Nematostella* **(Paper 3, Figure 1A)**. In Paper 3, we introduce the preliminary results of a first analysis of this dataset focusing on cell types involved in extra- and intracellular digestion.

Using a MARS-Seq approach, the single cell transcriptomes of 6767 cells were bioinformatically grouped into 41 distinct clusters characterized by unique sets of marker genes **(Paper 3, Figure 1B)**. Using ISH, we were able to assign a putative identity to most clusters based on marker gene expression pattern, transcriptional

signatures, cell localization and morphology. First, we validated the clustering by identifying previously described cell types such as cnidocytes and retractor muscle **(Paper 3, Figure S2)**. Then, in order to test how our dataset relates to the previously published whole-organism atlas (Sebé-Pedrós et al. 2018), we merged both datasets and performed a new clustering analysis **(Paper 3, Figure S3A-C)**. We obtained 81 clusters of which many grouped cells originating from both datasets, therefore indicating the reduced likelihood of a strong batch effect influencing our results. This new analysis additionally allowed us to validate our tissue-specific approach: as expected, a cluster defined by the expression of the yolk precursor gene *vitellogenin* contained exclusively cells from our adult female mesentery dataset **(Paper 3, Figure S3D)**, while another cluster expressing the epidermal marker *tetraspanin* only contained cells from the whole-organism dataset **(Paper 3, Figure S3E)**.

Our mesentery dataset analysis allowed us to identify sub-populations within cell type families such as neurosensory (6 clusters) or neuroglandular cells (4 clusters), and we could detect a cluster corresponding to oocytes **(Paper 3, Figure 1B)**. Remarkably, however, the most diverse cell type in the *Nematostella* mesentery are gland cells with 12 clusters likely representing each a different gland cell type **(Paper 3, Figure 1B)**. The transcriptomic profiles of these clusters revealed a high diversity of identifiable digestive gland cells (6 clusters) expressing specific sets of extracellular digestive enzymes. We notably identified three clusters expressing a selection of *trypsin-like* genes **(Paper 3, Figure 2A)**, and three clusters specialized respectively in the expression of *phospholipases* **(Paper 3, Figure 2B)**, *chitotriosidases* **(Paper 3, Figure 2C)** or other peptidases **(Paper 3, Figure 2D)**. *In situ* hybridization experiments using marker genes for these cell populations confirmed the previously reported location of gland cells in the cnidoglandular tract, and highlighted their typically elongated and vesicle-rich morphology **(Paper 3, Figure 2E-H)**.

After ingested food has been broken down extracellularly under the action of digestive enzymes, food particles are taken up by cells of the mesentery **(Paper 1, Figures 1 and 3)**. In our scRNA-seq dataset, we could identify three cell clusters expressing a variety of endocytosis and lysosomal marker genes, and thus representing three putative trophic

endocyte cell types **(Paper 3, Figure 3A)**. These include endocytosis receptor genes (e.g. *mannose receptor*, *ldlr*), actin remodeling genes (e.g. *elmo*, *rhoA*) and *clathrin* genes. The role of these cells in intracellular digestion was corroborated by the expression of lysosomal enzyme genes, such as *cathepsins* and *lysosomal lipases* **(Paper 3, Figure 3B)**. The spatial expression patterns of endocytic marker genes revealed that cells from all three clusters are located in the intermediate tract of the septal filament **(Paper 3, Figure 3C-E)**. In addition, two clusters appear to group cells located in the trophic tract while the third one is represented by cells from the gonad epithelium **(Paper 3, Figure 3E)**. Overall, these results further confirm our previous observations **(Paper 1)** on a single cell transcriptomic level and allow for a deeper characterization of digestive cells on the molecular level.

Chapter 3: Discussion

The goal of the present thesis was to shed light on the evolution of the cells and molecules underlying two mechanisms linked to energy homeostasis in animals: (1) the uptake and digestion of food, which allows organisms to acquire and store energy, and (2) vitellogenesis, during which maturing oocytes act as a nutrient sink. We present here the first study to combine physiological and molecular approaches to investigate these mechanisms in a non-bilaterian animal, the sea anemone *Nematostella vectensis*.

1. Intracellular digestion, an ancestral feeding mode

The uptake of particles through endocytic mechanisms (e.g. receptor-mediated endocytosis, phagocytosis) and subsequent intracellular digestion are highly conserved in eukaryotes, including in single-celled organisms such as choanoflagellates (Dayel and King 2014). Endocytosis is therefore believed to be the ancestral feeding mode in metazoans (Yutin et al. 2009; Dayel and King 2014). Among animals, this process has however so far been mainly studied in vertebrates and insects in the context of innate immunity (Rabinovitch 1995; Gordon 2016; Nazario-Toole and Wu 2017; Rosales and Uribe-Querol 2017). A major aspect of this thesis therefore aimed at characterizing nutritive endocytosis and intracellular digestion on the physiological and molecular levels in the cnidarian *Nematostella vectensis*, in order to better understand the evolution of endocytic cell types in animals.

1.1 Bilaterian particle uptake mechanisms are conserved in Nematostella

1.1.1 The regionalized gastric cavity of anthozoans

The gastric cavity of anthozoans is not a uniform, sack-like blind gut, as found for example in hydrozoans, but is compartmentalized by complex gastrodermal folds with functions in nutrition and reproduction: the mesenteries (Fautin and Mariscal 1991; Brusca et al. 2016). In **Paper 1**, we show that three specific regions of adult female mesenteries are involved in the uptake of BSA-coated latex beads: the intermediate tract of the septal filament, the somatic epithelium of the gonad (which surrounds immature oocytes) and the aboral trophic tract. Moreover, we describe in **Paper 2** that these three

regions are not only taking up particulate matter but are also involved in the absorption of fatty acids delivered in solution to the gastric cavity, adding more support to their prominent nutritive role in *Nematostella*.

Previous studies using particle and dissolved nutrient uptake assays conducted in the sea anemone *Actinia equina* pointed to the intermediate tract as the main endocytic region in the anthozoan gastric cavity (Van-Praët 1978, 1980). Using petroleum and cod liver oil, the same author found that lipid uptake also occurred in this structure as well as in other unspecified regions of the mesenteries. These likely correspond to the previously uncharacterized region that we have newly termed 'trophic tract' (Van-Praët 1980). A similar degree of regionalization of endocytic and absorptive activities has not been observed in other cnidarian groups and currently seems to be found only in anthozoans. In hydrozoan and cubozoan polyps, the gastric cavity is not compartmentalized and endocytic cells are distributed in the gastrodermis all along the body column (Haynes and Davis 1969; Lesh-Laurie 1991; Bouillon et al. 2006). A similarly wide distribution of endocytes is found in the medusa life stage of hydrozoans and scyphozoans, although a higher endocytosis activity has been reported in the gonad region of *Aurelia aurita* (Scyphozoa) and in the gonad region and tentacle bulbs of *Clytia hemisphaerica* (Hydrozoa) (Lesh-Laurie 1991; Arai 1996; Bouillon et al. 2006; Amiel et al. 2010).

The high endocytic activity observed in the intermediate tract is intimately linked to the adjacent ciliated tract, which creates a water flow causing the accumulation of particles and macromolecules (see mesentery morphology Figure 7C). Why is it, however, that the rest of the gastrodermis does not equally participate in food uptake in *Nematostella*? I hypothesize that the presence of specialized endocytic regions increases the efficiency of other physiological processes that are also tied to the regionalization of the mesentery, namely vitellogenesis and nutrient storage. We have shown in **Paper 2** that fatty acids absorbed by the epithelial cells of the gonads were rapidly transported through the epithelium and incorporated in vitellogenic oocytes. Additionally, previous work in juvenile polyps has found the trophic tract to be a nutrient storage tissue (Steinmetz et al. 2017), and large lipid reserves were also observed in this structure in adults. The proximity between endocytic cells and nutrient sinks reduces the necessity and distances

for nutrient transport, therefore representing a parsimonious physiological solution. The mechanisms underlying vitellogenesis and the possibility of systemic nutrient transport from the trophic tract towards other parts of the body are further discussed in part 2 of this chapter.

1.1.2 Several conserved endocytic pathways at play in the mesentery

The size of internalized particles is a defining characteristic to distinguish between different endocytic pathways. Notably, phagocytosis mediates the uptake of large (> 0.5µm) particles, while smaller particles and macromolecules are likely to be taken up via receptor-mediated endocytosis or macropinocytosis. Therefore, the results of our micro- and nanoparticle uptake assays suggest the occurrence of at least two different endocytic pathways in *Nematostella*. This is further confirmed by the co-expression of marker genes for both phagocytosis and receptor-mediated endocytosis, which are remarkably conserved among animals, in the three endocytic regions of the mesentery (Yutin et al. 2009; Beljan et al. 2020). Examples include low-density lipoprotein receptors (LDLR, e.g. *vldlrA*, *lrp1*), GTPases of the Ras superfamily (*rab2*, *cdc42*, *rhoA*) that are key players in actin remodeling during vesicle trafficking, endo- and exocytosis (Beljan et al. 2020), or *clathrin* genes, which encode the scaffold proteins characteristic of clathrin-mediated endocytosis (McMahon and Boucrot 2011). Altogether, our results support phagocytosis and clathrin-mediated endocytosis, two endocytic pathways highly conserved among animals, as the main endocytic pathways underlying food uptake in *Nematostella*. Recently, macropinocytosis has been claimed to occur ubiquitously in the coral *Stylophora pistillata*, but the study did not include mesenteries (Ganot et al. 2020). The authors also suggest that macropinocytosis occurs in most anthozoans. Our current assays do not allow us to assess this assumption in *Nematostella* as it is challenging to distinguish between micropinocytosis and receptor-mediated endocytosis: their particle size range overlap, and specific macropinocytosis marker genes are lacking. Combining particle uptake assays with macropinocytosis-specific inhibitors (e.g. Phosphoinositide 3-kinase inhibitor Wortmannin; Williams & Kay, 2018) will be necessary to answer that question.

Interestingly, while the gastric cavity displays a clear regionalization with regards to food uptake, the endocytic regions do not appear to show any further functional or spatial segregation. We found that the intermediate tract, gonad epithelium and trophic tract internalize both large (1µm) and small (20nm) particles as well as macromolecules, suggesting the co-occurrence of several modes of particle uptake in all endocytic regions. Two different scenarios may explain this observation: either we cannot currently distinguish between distinct specialized populations of trophic endocytes taking up specific types of cargoes within one endocytic region, or several uptake mechanisms co-occur within the same cells. Considering that all cells of the intermediate tract seemed to take up each of the products tested in our assays, and the uniform expression patterns of endocytosis-related genes in all three regions, our results indicate that phagocytosis and receptor-mediated endocytosis are likely to co-occur in the same cells. This assumption is also supported by the single cell sequencing dataset in which endocytic cell types did not display mechanism-specific transcriptomes (see 1.2.2). Future work will investigate the potential co-occurrence of endocytic mechanisms, including macropinocytosis, in single cells of the three endocytic regions. Taking advantage of the well-characterized ultrastructural features of each of these particle uptake modalities, we plan on investigating the ultrastructure of endocytic cell types after incubation with various substrates using transmission electron microscopy, allowing for the direct visualization of the internalization process.

1.2 Uncovering the diversity of digestive cell types in a cnidarian model

A recent whole-organism single-cell RNA sequencing (scRNA-seq) study conducted on juvenile *Nematostella* polyps (Sebé-Pedrós et al. 2018) demonstrated that a surprisingly large number of cell types are present in this species, questioning the historical belief that a ‘simple’ body plan such as that of cnidarians is reflected by a low cell type diversity (Valentine 2003). In **Paper 3**, we used the same scRNA-seq approach to sequence single cells of the mesenteries of adult female polyps and observed an accordingly high diversity of putative digestive cell types in *Nematostella*.

1.2.1 Specialized gland cells are responsible for extracellular digestion

The scRNA-sequencing of whole polyps revealed a large number of putative gland cells (Sebé-Pedrós et al. 2018), and this cell type family was the most represented in our mesentery dataset. Upon further analysis of transcriptional profiles, it appeared that at least 6 of these clusters correspond to digestive gland cells, each characterized by the expression of a specific set of extracellular digestive enzyme genes (e.g. *trypsin*, *carboxypeptidase*, *chitotriosidase*). All digestive gland cells were localized in the cnidoglandular tract of the septal filament, a region of the mesentery previously known to be composed of gland cells and cnidocytes and to play a role in extracellular digestion in anthozoans (Van-Praët 1985; Fautin and Mariscal 1991). Moreover, most digestive gland cells were concentrated in the aboral region of the mesentery. This was previously observed in *A. equina* (Van Praët 1982) and in the coral *S. pistillata* (Raz-Bahat et al. 2017), indicating that the septal filaments in anthozoans are also regionalized along the oral-aboral axis with regards to extracellular digestion.

Ultrastructural studies described the occurrence of several distinct types of zymogen cells in sea anemones (Van-Praët 1985; Shick 1991), and double color *in situ* hybridizations showed the mutually exclusive expression of *chitotriosidase/chitinase* and *trypsin* genes in cells of the cnidoglandular tract in juvenile *Nematostella* polyps (Steinmetz et al. 2017). Indeed, our scRNA-seq analysis allowed us to differentiate and classify the digestive gland cells of the mesentery based on their enzyme repertoires into four groups: we identified trypsinergic gland cells, Phospholipase producers, Chitinase producers and producers of other peptidases (e.g. Carboxypeptidases). Interestingly, the presence of specialized digestive gland cells is not an anthozoan peculiarity: 5 distinct glandular cell types involved in extracellular digestion were similarly described in the hydrozoan medusa *Clytia hemisphaerica* (Chari et al. 2021). Future work will reveal if the gland cell diversity seen in anthozoans and hydrozoans reflects an ancestral situation or has occurred by convergent evolution in different cnidarian phyla.

The production of digestive enzymes in the animal gut is energetically costly, and is therefore tightly regulated. In bilaterians possessing a regionalized through-gut, the

expression of extracellular digestive enzymes is restricted to specific segments of the digestive tract: for example, the expression of sugar-degrading glycosidases occurs in anterior regions of the *Drosophila* midgut, while peptidases are expressed more posteriorly (Dutta et al. 2015). Consistent with these results, 15 populations of enterocytes, some characterized by specific enzymatic repertoires, were identified in a scRNA-seq study of the fly midgut (Hung et al. 2020). While such a division of labor with regards to extracellular digestive enzyme production is coherent in the context of sequential digestion in a regionalized gut, its relevance in a sack-like gastric cavity is less obvious. A prevalent hypothesis suggests that enzyme duplication events drove the diversification of gland cells in animals (Brückner and Parker 2020). This theory is supported by a genomic analysis of the *Nematostella* tryptome that revealed a wide diversification of *trypsin* genes linked to gene duplications and domain acquisitions (Babonis et al. 2019). The emergence of specialized gland cell types in the context of cnidarian digestion might have allowed for subtle variations in the digestive process in the absence of a morphological regionalization of the gut. Examples of such variations are the temporal regulation of enzyme production, or the activation of a subset of gland cells depending on the composition of the ingested food.

1.2.2 Single-cell transcriptomics highlights three distinct populations of endocytic cells

Three cell clusters in our scRNA-seq dataset were annotated as putative trophic endocytes based on the expression of genes typically involved in phagocytosis, receptor-mediated endocytosis and lysosomal digestion. ISH of marker genes revealed that these three clusters represent cells located in the intermediate tract, gonad epithelium and trophic tract of the mesentery, overall matching the distribution of highly endocytic regions previously described (see **Papers 1** and **2**) and thus supporting their role in intracellular digestion.

Based on the observation that the endocytic regions of the mesenteries use at least two different endocytic pathways, we hypothesized that this could be explained either by the presence of several, intermingled endocytic cell types or by the co-occurrence of

different endocytosis modes in the same cells (see section 1.1.2 of the present chapter). Our scRNA-seq results bring a new perspective to this question and indicate that these hypotheses are not mutually exclusive: while three distinct populations of trophic endocytes are apparent in the endocytic regions of the mesentery, all of these cells express genes supporting both clathrin-mediated endocytosis (e.g. *clathrin heavy* and *light chain* genes) and phagocytosis (e.g. *rhoA*). What then are the differences between these three endocytic cell types?

Two of these clusters display a relatively strong expression of partially overlapping sets of endocytosis markers and lysosomal enzyme genes, supporting a prevalent role in intracellular digestion. However, while one cluster is broadly spread over the intermediate and trophic tract, the other one locates mainly in a small subset of cells within the trophic tract. The reason for this cellular subdivision of the trophic tract is unclear, and cannot be deducted readily from the transcriptomic analysis of both cell clusters. Further work, including additional ISH to demarcate the precise localization of both sub-populations of cells and ultrastructural comparisons are necessary to elucidate the precise role of these cells during intracellular digestion. The third identified trophic endocyte cell population presents more distinct features, including a characteristic localization in the gonad epithelium which harbors vitellogenin-producing cells (Levitan et al. 2015). This confirms previous observations of particle and nutrient uptake in this region, and strongly suggest a dual role in nutrition and vitellogenesis for these cells.

Additionally, all three trophic endocytes populations appear to be present in the intermediate tract, which was previously shown to carry a nutritive function (Van-Praët 1978, **Paper 1**). Further experiments are needed to investigate the differences between the three cell types and the potential functional advantages resulting from their colocalization.

1.3 A common evolutionary origin for trophic endocytes and innate immune cells?

Although feeding is likely the ancestral function of phagocytosis (Yutin et al. 2009; Dayel and King 2014), this endocytic pathway is also used by innate immune cells (e.g.

vertebrate macrophages) to clear the body of pathogens, undesirable particles and apoptotic cells (reviewed in Gordon, 2016; Rosales & Uribe-Querol, 2017). The molecular mechanisms and cell types involved in the phagocytosis of food particles or pathogens share many similarities, prompting the hypothesis of a shared evolutionary origin of trophic and immune phagocytes (Broderick 2015; Hartenstein and Martinez 2019). These similarities range from the consistency of ultrastructural processes, regardless of the nature of the cargo, to shared receptors and enzymes responsible for particle recognition and degradation (Rougerie et al. 2013; Broderick 2015; Lancaster et al. 2019). In fact, some cell types (e.g. sponge archaeocytes) engage in both immune and nutritive phagocytosis (Imsiecke 1993; Funayama et al. 2005). In *Nematostella*, the boundaries between nutrition and defense against exogenous material and organisms in the gastric cavity are similarly unclear. We have shown that the trophic endocytes of the mesentery readily take up inactivated *E. coli* particles, which could represent an immune response although it is unknown whether bacteria are also a part of the diet in this species. The molecular machinery underlying phagocytosis appears highly conserved between *Nematostella* and bilaterians, as discussed previously, and includes genes typically involved in the innate immune response in vertebrates. We have notably shown expression of the *LBP/BPI* gene, which binds bacterial lipopolysaccharide and triggers the phagocytosis of pathogens in mammals (Grunwald et al. 1996; Weiss 2003), in endocytic regions of the *Nematostella* mesenteries. We also found an ortholog of the *mannose receptor* gene, encoding a pattern recognition receptor in vertebrate macrophages, expressed in the same regions (Gazi and Martinez-Pomares 2009). Similarly expressed in the *Nematostella* mesentery, the actin-remodeling gene *elmo* is involved in the phagocytic removal of apoptotic cells from *C. elegans* to mammals (Gumienny et al. 2001; Park et al. 2007). In addition, the array of intracellular digestive enzyme genes identified in trophic endocytes (e.g. *cathepsins*, *lipases*) is also responsible for the breakdown of pathogens in the lysosome of immune cells (Broderick 2015; Gray and Botelho 2017).

Further comparisons of bilaterian endocytic cell types involved in nutrition with immune phagocytes is however hindered by the lack of studies focusing on trophic endocytic cells. To the best of our knowledge, molecular studies on trophic phagocytes in

bilaterians have only been conducted in planarians and acoels. Planarian gastric phagocytes have been shown to express a range of endocytosis-related genes similar to that observed in *Nematostella* (e.g. *rhoA*, *cdc42*, *clathrin*), as well as the transcription factor *nkx2.2* which is typically expressed in endoderm-derived enteroendocrine cells of the mammalian gut (Forsthoefel et al. 2012; Gross et al. 2015). Another planarian study showed however that phagocytes of the gastric cavity express *foxF1*, which is a marker for the lateral plate mesoderm of vertebrates (Scimone et al. 2018; Prummel et al. 2020). In acoels, a recent publication of the scRNA-seq transcriptome presented a description of putative intracellular digestive cell types based on lysosomal marker genes, without revealing their distinct transcription factor profiles (Duruz et al. 2021). Altogether, the scarcity of data makes a comparison of immune and trophic phagocytic cell types difficult even among bilaterians.

Nevertheless, we can make a first try to compare trophic endocytes with developmental regions or cell types in bilaterians. Interestingly, we found that the transcription factors *foxC* and *six4/5* are shared among endocytic regions of the mesentery in *Nematostella*. The combination of these transcription factors (together with *nkx3*/*bagpipe* expressed in the juvenile trophic tract; Steinmetz et al. 2017) is reminiscent of the ventro-lateral mesoderm of vertebrates and insects (Azpiazu and Frasch 1993; Evans et al. 1995; Topczewska et al. 2001; Clark et al. 2006). This surprising transcription factor profile not only highlights a ‘mesodermal affinity’ of trophic endocytes in *Nematostella*, but also suggests a possible similarity with phagocytic cells of mesodermal origin, such as the innate immune cells of insects and vertebrates, or the coelomocytes of echinoderms, nematodes and annelids (Herbomel et al. 1999; Evans et al. 2003; Amin et al. 2010; Smith et al. 2018; Engelmann et al. 2018).

Overall, we provide a first account of the general transcriptome and transcription factor profile of trophic endocytes in a non-bilaterian organism. This sets the basis for future comparative studies between phagocytic cell types in animals. Specifically, a deeper analysis and larger taxon sampling among bilaterians possessing trophic phagocytes (e.g. amphioxus, acoelomorphs) is necessary to better understand the relationship between immune and nutritive cell types.

2. Investigating the fate of dietary nutrients in *Nematostella*: trans-epithelial lipid transport during vitellogenesis

Digested dietary nutrients in bilaterians are usually transported from the digestive cells towards other parts of the body where they support metabolism or are stored for future use. In order to elucidate the fate of nutrients digested intracellularly in the endocytic regions of the *Nematostella* mesentery, we studied vitellogenesis as a paradigm for this process in a cnidarian. Vitellogenesis, the accumulation of nutrients in growing oocytes, typically involves the transport of dietary lipids. Our results, interpreted from an evolutionary perspective, shed light on the mechanisms of vitellogenesis in anthozoans and on the molecular and cellular evolution of lipid transport pathways in animals.

2.1 Modalities of vitellogenesis in a cnidarian model

2.1.1 Vitellogenesis is mainly heterosynthetic in Nematostella

After establishing the prevalent role of the gonad epithelium in food uptake in **Paper 1**, we show in **Paper 2** the fast trans-epithelial transport (< 2 hours) of a dietary lipid tracer from the gastric cavity into growing oocytes. Additionally, we confirmed previously published high expression levels of *vitellogenin*, a highly conserved yolk precursor gene abundant in *Nematostella* eggs (Lotan 2014), in the gonad epithelium surrounding the oocytes (Levitan et al. 2015). Our observations are also consistent with previous studies claiming the prevalence of heterosynthetic vitellogenesis in cnidarians, i.e. the production of yolk precursors in somatic tissue and their subsequent transfer into the eggs (Schechtman 1955). This hypothesis was based both on ultrastructural data, which showed the presence of numerous endocytic pits on the surface of the oolemma (Eckelbarger and Larson 1988, 1992; Eckelbarger et al. 1998), including in *Nematostella* (Eckelbarger et al. 2008), and on the production of yolk precursors in somatic cells (Shikina et al. 2013, 2015; Levitan et al. 2015).

The gonads of mature female *Nematostella* polyps fed consistently in the laboratory contain oocytes at all stages of maturation at all times (Eckelbarger et al. 2008), and spawning can be sustainably induced every second week for years on end. Moreover, vitellogenesis in anthozoans is believed to be initiated as soon as small oocytes bulge

from the gastrodermis into the mesoglea, suggesting that it likely takes place for the majority of the time during which an egg is present in the gonad (Shick 1991; Eckelbarger et al. 2008; Shikina et al. 2013). Such consistent rates of oocyte maturation during the reproductive life of the organism come with high nutritive requirements, and the direct and quick trans-epithelial transport of dietary nutrients from the gastric cavity into the oocytes is therefore consistent with this reproductive strategy (see Eckelbarger 1994 for a review on the correlation between vitellogenesis modes and animal life histories). Our observations do not exclude the possibility that autosynthesis, the endogenous production of yolk by the oocytes, takes place concurrently to heterosynthesis in *Nematostella*. However, as it appears that the oocytes in this species contain large amounts of heterosynthetic yolk (67% of the total protein content of ovulated eggs, Lotan et al. 2014) and as autosynthetic vitellogenesis is associated with increased maturation time and slower egg production rates (Eckelbarger 1994), it seems unlikely that autosynthesis contributes significantly to vitellogenesis in *Nematostella*.

2.1.2 A role for gonad accessory cells in lipid transport during vitellogenesis

It is tempting to draw parallels between the gonad epithelium in *Nematostella* and the ovarian follicle cells which surround the gametes in many animal groups, including in vertebrates where they are known to trigger ovulation through hormone secretion (Polzonetti-Magni et al. 2004). According to Wourms (1987), who reviewed the literature to establish a consensual definition of this polymorphic cell type, follicle cells are of somatic origin and do not maintain cytoplasmic bridges with the oocytes. They are considered to perform at least one of the following functions: (1) provide mechanical support or protection to the oocytes, (2) produce secondary envelopes around the oocytes, (3) produce metabolites and yolk precursors, (4) resorb apoptotic oocytes (Wourms 1987). Cells of the gonad epithelium in *Nematostella* indeed match several of these criteria. They are of somatic origin and an ultrastructural study showed no evidence of cytoplasmic bridges with the oocytes (Eckelbarger et al. 2008). Functionally, the anthozoan gonad epithelium surrounds growing oocytes, providing mechanical support, and contributes to the production of yolk precursors (Levitan et al. 2015; **Paper 2**) and protective mucus enveloping the eggs before spawning (Frank and

Bleakney 1976; Uhlinger 1997). In cnidarians, follicle cells have been described in scyphozoans (Eckelbarger and Larson 1993; Tiemann and Jarms 2010) and pennatulaceans (sea pens, Anthozoa) (Eckelbarger et al. 1998). They have not however been formally identified in a previous publication describing the ultrastructure of oogenesis in *Nematostella* (Eckelbarger et al. 2008). Based on the strong structural and functional similarities with follicle cells described in bilaterians and other cnidarians, I propose for the gonad epithelium in *Nematostella* to be considered a follicular tissue.

The same ultrastructural study postulated that during vitellogenesis in *Nematostella*, nutrients are partly taken up by the oocytes from the mesoglea through endocytosis, and partly channeled from the gastric cavity into the eggs via the trophonema (Eckelbarger et al. 2008). Our results only partially support these findings: we did not observe any uptake of food particles, fatty acids or glucose (data not shown) in trophonemata, challenging their proposed nutritive role (Larkman and Carter 1982; Fautin and Mariscal 1991; Shick 1991). The presence of a thin mesoglea layer between the epithelium and the oocytes suggests that lipid transport may occur through the extracellular matrix, possibly through mesogleal pores which have been described in *Hydra* (Shimizu et al. 2008).

Accessory cells associated with growing oocytes have been described both in bilaterian (reviewed in Polzonetti-Magni et al. 2004; Brusca et al. 2016) and non-bilaterian groups including cnidarians (Eckelbarger and Larson 1993; Eckelbarger et al. 1998; Tiemann and Jarms 2010) and sponges (Leys and Degnan 2005; Degnan et al. 2015). First thought to be restricted to vertebrates and insects (Eckelbarger 1994), heterosynthetic vitellogenesis has also been found in cnidarians (Eckelbarger 1979; Eckelbarger et al. 2008; Shikina et al. 2013, 2015; Levitan et al. 2015), sponges (which also possess nutritive nurse-like cells; Adiyodi and Adiyodi 1983; Wourms 1987) and ctenophores (Hernandez-Nicaise 1991). These observations, supported by the present work, suggests that the last common ancestor of bilaterians and cnidarians may have used heterosynthetic yolk production, which may have been mediated by accessory cells similar to the follicle cells found in cnidarians and bilaterians.

2.2 Conserved lipoprotein-lipoprotein receptor pairs mediate lipid transport in Nematostella

Lipid transport in animals is mediated by proteins of the Large Lipid Transfer Proteins (LLTP) family, which is conserved from placozoans to vertebrates (Babin et al. 1999; Smolenaars et al. 2007; Wu et al. 2013). Here, we investigated the potential roles of two LLTPs found in *Nematostella*: the yolk precursor Vitellogenin and an ortholog of vertebrate ApolipoproteinB (ApoB) and insect Apolipophorins, which are involved in systemic lipid transport (including vitellogenesis) in bilaterians (Smolenaars et al. 2007; Voet et al. 2016).

2.2.1 Vitellogenin uptake into the oocytes via VLDLR-mediated endocytosis

Vitellogenin, a specialized member of the LLTP family, plays a key role as a yolk precursor in all major animal groups except eutherian mammals (Babin et al. 1999; Smolenaars et al. 2007; Riesgo et al. 2014). The *Nematostella vitellogenin* gene is expressed exclusively in the female somatic gonad epithelium (Levitan et al. 2015; **Paper 2**) while the Vtg protein was shown to be the most abundant protein in the mature eggs (Lotan et al. 2014), suggesting that it is transported from the gonad epithelium into the oocytes. In addition, the protein domain structure of *Nematostella* Vitellogenin is identical to the bilaterian protein (Hayward et al. 2010). Altogether, this suggests a conserved role as a yolk precursor in this species.

In bilaterians, Vitellogenin synthesized in extra-ovarian tissues (e.g. vertebrate liver, hepatopancreas in crustaceans, intestinal cells in nematodes; Adiyodi and Adiyodi 1983; Wallace 1985) and/or in ovarian accessory cells (e.g. follicle cells in *Drosophila* or *X. laevis*; Brennan et al. 1982; Wallace 1985) is internalized into the oocytes via LDL receptor-mediated endocytosis (Kawooya and Law 1988; Raikhel and Dhadialla 1992; Subramoniam 2011). The chicken and insect Vitellogenin receptor, as well as the insect Lipophorin receptor (also involved in vitellogenesis) are all orthologous to the vertebrate VLDLR (Nimpf and Schneider 1991; Schonbaum et al. 1995; Dantuma et al. 1999). In *Nematostella*, three endocytosis receptors of the LDL family (VldlrA, VldlrB, Lrp1) were previously identified in the proteome of mature eggs (Lotan et al. 2014). We found

them to be specifically expressed in oocytes and, according to our phylogenetic analysis, to be strikingly orthologous to Very Low-Density Lipoprotein Receptors (VLDLR) conserved in bilaterian Vitellogenin transport. Their expression in the *Nematostella* oocytes also matches the numerous endocytic pits found on the oolemma in a previous ultrastructural study (Eckelbarger et al. 2008). Altogether, this strongly suggest that all three VLDL receptor orthologs found in the oocytes in *Nematostella* contribute to the accumulation of yolk during vitellogenesis and may be Vitellogenin receptors in this species.This finding suggests that a ligand-receptor pair, consisting of Vitellogenin and VLDL receptors, is conserved between cnidarians and bilaterians. Further experiments are needed to validate this hypothesis and the molecular modalities of vitellogenesis in *Nematostella*, including for example the knock-down of putative Vitellogenin receptors genes in growing oocytes.

Proteins of the LLTP family, including Vitellogenin, form a hydrophilic shell around a lipid-rich core. This allows the transport of lipids through the extracellular environment such as the blood stream (Voet et al. 2016). Since Vitellogenin has been shown to contain a large proportion of fatty acids (Silversand and Haux 1995; Li and Zhang 2017), it remains to be tested if the fatty acid tracer used in our lipid uptake experiments and ultimately transported into the maturing oocytes in *Nematostella* is incorporated into Vitellogenin lipoproteins. In that case, our assay would effectively allow us to observe the vitellogenic process from the ingestion of dietary lipids to their incorporation in a yolk precursor, and ultimate transfer into the oocytes. To investigate this hypothesis, future work will focus on establishing a Vitellogenin protein fusion reporter line.

2.2.2 A role for ApoB in spermatogenesis?

A second LLTP identified in *Nematostella* was previously annotated as a putative Vitellogenin despite a phylogenetic analysis identifying it as an Apolipoprotein (Hayward et al. 2010). Although the two proteins share identical domain structures, which prompted this classification, our phylogenetic analysis confirmed it as a clear ortholog to the vertebrate ApoB and insect Apolipophorin proteins (Hayward et al. 2010). Both ApoB and apolipophorins play a major role in systemic lipid transport,

distributing lipid throughout the body via the circulatory system (Palm et al. 2012; Voet et al. 2016). Apolipophorins are additionally involved in lipid transport during insect vitellogenesis (Kawooya and Law 1988; Raikhel and Dhadialla 1992). The Vitellogenin-like domain structure and expression of the *Nematostella apoB* ortholog in the gonad epithelium suggested that it might be involved in vitellogenesis in this species. In order to test this hypothesis, we generated a fluorescent fusion protein reporter line, which to our surprise showed no apparent protein levels in the growing oocytes. ApoB was however detected in the proteome of mature eggs, although at much lower levels than Vitellogenin (2000-fold lower concentration) (Lotan et al. 2014). This indicates that it may play a minor role during vitellogenesis and that its concentration lies below the detection limit of immunofluorescence.

Strikingly, however, the ApoB fusion protein appeared to be prominently present in the male gonad where it was detected in vesicles associated with individual maturing sperm cells. The signal was particularly strong in the regions of the spermaries containing newly differentiated cells, suggesting that ApoB might be involved in spermatogenesis from early stages of maturation in *Nematostella*. Two hypotheses could explain the presence of ApoB in the spermaries. First, ApoB could play a role in the maturation of sperm cells in *Nematostella* by providing the building blocks of new cellular membranes. ApoB in mammals plays an important role in the systemic transport of cholesterol, which greatly influences the maturation of sperm cells in this group (reviewed in Whitfield et al. 2015). Notably, male ApoB knockout mice present a reduced fertility coupled with decreased cholesterol plasma levels (Huang et al. 1996). Research investigating the precise role of ApoB in male gametogenesis is however lacking, and the prevalence and role of cholesterol in *Nematostella* is mostly unknown. A second hypothesis is that ApoB is responsible for the accumulation of lipids in sperm cells as energy storage, not unlike vitellogenesis. Endogenous energy storage in sperm cells appears to be a frequent strategy in animals spawning in open waters, where spermatozoa might travel over longer distances and need to persist for longer periods of time (Bishop 1962). This has been shown for example in sea urchins where the midpiece of spermatozoa in *Arbacia lixula* and *Paracentrotus lividus* contains lipid droplets that are believed to provide the energy necessary for swimming (Mita et al. 1994; Mita and

Nakamura 1998). Considering the stronger ApoB signal observed in early developing spermatozoa compared to mature cells, a role in membrane formation appears more likely than in the storage of lipids. These functions are however not mutually exclusive, and more work is needed to validate our hypotheses.

2.2.3 Could systemic lipid transport via the extra-cellular matrix occur in a cnidarian?

In general, systemic lipid transport in bilaterians is mediated by lipoproteins, LLTP-lipid complexes that travel in the circulatory system and are taken up by target cells through LDL receptor-mediated endocytosis (Babin et al. 1999; Dieckmann et al. 2010; Voet et al. 2016). So far in this work, we have discussed the presence of conserved LLTP lipoprotein-LDL receptors in a cnidarian model in the context of gametogenesis. The molecular pathways underlying vitellogenesis are very conserved in animals and very similar to those underlying systemic lipid transport, independently of the localization of yolk production (i.e. ovarian or extra-ovarian) and of the presence of a developed circulatory system (Wourms 1987; Raikhel and Dhadialla 1992; Babin et al. 1999; Smolenaars et al. 2007). It is therefore all the more fascinating that we identified in *Nematostella* specific orthologs of ApoB and Apolipophorin proteins, which are major systemic lipid transporters in vertebrates and insects, respectively. This raises a question that may have important implications for the evolution of physiological systems in animals: could systemic lipid transport occur in cnidarians, which appear to lack an extracellular matrix-based or endothelial circulatory system?

In *Nematostella*, the trophic tract is a very interesting structure to look at in this context: it expresses *apoB*, shows high levels of ApoB-PSmOrange fusion protein, and is one of the major tissues taking up fatty acids in our uptake assays. In addition, it was shown to be a major lipid storage tissue both in juvenile polyps (Steinmetz et al. 2017) and in adults (**Paper 2**). To the best of our knowledge, this region of the mesentery displays no functions other than endocytosis and nutrient storage. The trophic tract is in addition located rather remotely from the gonad and head structures, making it plausible that nutrients taken up and stored there could be transported towards other regions of the

body, especially when nutritional resources are scarce. Strikingly, supporting this hypothesis, our fatty acid uptake experiments revealed the presence of lipid-containing droplets located within the mesoglea of the trophic tract after a one-week chase.

It is unfortunately still unclear whether the fatty acid-containing particles detected in the mesoglea were contained inside cells, or were freely moving in the mesoglea, like lipoproteins in the bilaterian circulatory system. Most cnidarians (with the exception of hydrozoans) possess amoebocytes, a motile phagocytic cell type located in the extracellular matrix involved in immunity (Fautin and Mariscal 1991; Mydlarz et al. 2016). In sea anemones, amoebocytes have also been shown to take up radiolabeled amino acids and to contain lipid droplets, suggesting that they may indeed be involved in nutrient transport (Young 1974; Van-Praët 1978, 1980; Larkman 1984). Such transport of nutrients throughout the body by cells moving within the extracellular matrix would be reminiscent of the sponge archeocytes, which participate in the distribution of food taken up by choanocytes (Brusca et al. 2016). I believe, however, that the presence of fatty acid tracer in the extracellular matrix could also be explained by a vesicular lipid transport in the mesoglea. The transport of vesicles (e.g. exosomes) in the extracellular matrix, enhanced by extracellular fluids permeating collagen fibers, has previously been described in mammals (Huleihel et al. 2016; Lenzini et al. 2020).

Overall, our results support the possibility of systemic lipid transport events occurring in the mesoglea in *Nematostella,* but further work is needed to corroborate this assumption. We have generated a transgenic line by crossing the ApoB-PSmOrange line with an Ef1a-membraneGFP line, which allows us to visualize the localization of ApoB in animals where all cell membranes are labeled with GFP. Future experiments will include testing the co-localization of ApoB with fatty acid tracer molecules in this new transgenic line, which will elucidate the precise localization of fatty acids and, potentially, of ApoB in the mesoglea. More generally, functional gene analysis will be necessary to address the role of bilaterian lipid transporter orthologs in cnidarians.

3. Conclusions

In the present study, we were able to trace the path of dietary nutrients from their

digestion by cells of the mesenteries to their incorporation in vitellogenic oocytes in the sea anemone *Nematostella vectensis*. This represents the first extensive study of intracellular digestion and lipid transport on the physiological and molecular levels in a non-bilaterian animal, and sheds light on the evolution of these processes in animals.

Our research revealed the striking regionalization of the apparently simple gastric cavity of a cnidarian, reflected by a large number of cell types involved in digestion and reproduction in the mesenteries. We characterized the endocytic cell types in *Nematostella*, which take up food particles using a conserved physiological and molecular machinery that they share with phagocytic cells of the bilaterian innate immune system. After the food has been processed, we observed that dietary lipids are transported into the maturing oocytes via a conserved lipoprotein-lipoprotein receptor pair. Finally, we investigated the potential role of a bilaterian systemic lipid transporter ortholog in *Nematostella*, providing preliminary support for nutrient transport through the extracellular matrix in this species.

Our findings in *Nematostella* provide valuable insights to infer the ancestral modalities of food uptake, nutrient transport and vitellogenesis in the last common ancestor to bilaterians and cnidarians, yet many questions remain open. These include the evolutionary relationship between endocytic cell types fulfilling very different roles in nutrition and immunity in animals. Addressing this question will require a thorough molecular characterization of trophic endocytes in additional bilaterian and non-bilaterian species. Our observations challenge the notion that there is no systemic nutrient transport apart from gastric movements in cnidarians, and a potential role for the mesoglea are a rudimentary lipid transport route remains to be investigated.
Finally, we propose *Nematostella* as an ideal model to study the signaling and endocrine pathways underlying the regulation of food uptake, storage and energy expenditure (e.g. during gametogenesis). These fascinating aspects of energy homeostasis have seldom been studied in non-bilaterians and are key to understanding the evolution of this process in animals.

Chapter 4: References

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