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VISIONS AND PERSPECTIVES

Going beyond a static picture: the apple snail *Pomacea canaliculata* can tell us the life history of molluscan hemocytes

D Malagoli

Department of Life Sciences, University of Modena and Reggio Emilia, Italy

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Abstract

More than 40 years of studies on molluscan immunity have revealed a complex and dynamic immune system endowed with multifunctional circulating cells, *i.e.,* hemocytes that are regulated by diverse signaling molecules. However, very little is known about the dynamic processes that drive hemocyte proliferation, differentiation, maturation, and senescence. Evidence reported here highlights how the apple snail *Pomacea canaliculata* is an extremely promising research organism that will provide answers to the numerous questions regarding the life-history of molluscan and lophotrochozoan hemocytes.

Key Words: Ampullariidae; Gastropoda; hemocyte maturation; immunity; invertebrate hematopoiesis; *Pomacea canaliculata*

Metazoans base their survival and reproductive success on the simultaneous functioning of different organ systems. While many organ systems have a well-defined location, clear anatomical borders, and a recognized physiological role, the immune system does not have a fixed anatomy, and its primary function is maintaining the equilibrium between the organism and all the microorganisms that live in or around it (Bosch, 2014; Bachère *et al*., 2015). This structure and function make it extremely difficult to identify and dissect the many activities that the immune system plays during homeostasis. Most researchers have focused on mechanisms that are activated when the organism is challenged by pathogens or stressful events (Malagoli *et al*., 2017).

Since the 1980s, numerous scientists have studied the immune system of invertebrates. They were principally attracted by its relative simplicity and the lack of components related to acquired immunity, such as lymphocytes and immunoglobulins. The main rationale for these analyses was to both clarify the functional basis of the innate component of the human immune system and to improve our understanding of the many pathologies resulting from the malfunctioning of the innate immune system (Notarangelo *et al*., 2009).

Surprisingly, the invertebrate immune system has been revealed to be much more complex than expected, changing experimental approaches and revising our current knowledge of the comparative immunology field. One of the main changes that has occurred in the last decade was the publication of numerous papers that revealed the high degree of complexity in the invertebrate immune system, in addition to the presence of highly variable molecules (Armitage and Brites, 2016; Doolittle, 2016; Oren *et al*., 2016). The involvement of these molecules in the immune response of invertebrates has yet to be understood, but it is undeniable that these molecules are strikingly similar to vertebrate antibodies (immunoglobulins), which are characterized by hypervariable regions. Among invertebrates, the most important and commonly studied model is *Drosophila melanogaster* (Buchon *et al*., 2014). However, several other research organisms are available and well-studied, mainly because of their economic relevance (such as crustaceans and bivalves [Smith *et al*., 2016; Bachère *et al*., 2015]) or their importance as vectors of parasitic diseases (*e.g.*, mosquitoes and some gastropods) (Choi *et al*., 2012; Adema *et al*., 2017).

Over the past few years, our research group has used a molluscan gastropod, *Pomacea canaliculata*, as a research organism. This organism has great potential in the comparative immunology field because it gathers together many point of interest mentioned above, and many biological features make it easy to maintain in the lab and work with (Fig. 1A). *P. canaliculata* (aka, the golden apple snail), is indexed among the most invasive species in the world (www.issg.org/worst100_species.html), and its fast and vast spread raised concerns for several reasons. In economic terms, *P. canaliculata* is a

Corresponding author:
Davide Malagoli
Department of Life Sciences
University of Modena and Reggio Emilia
Via Campi 213/D, 41125, Modena Italy
E-mail: davide.malagoli@unimore.it

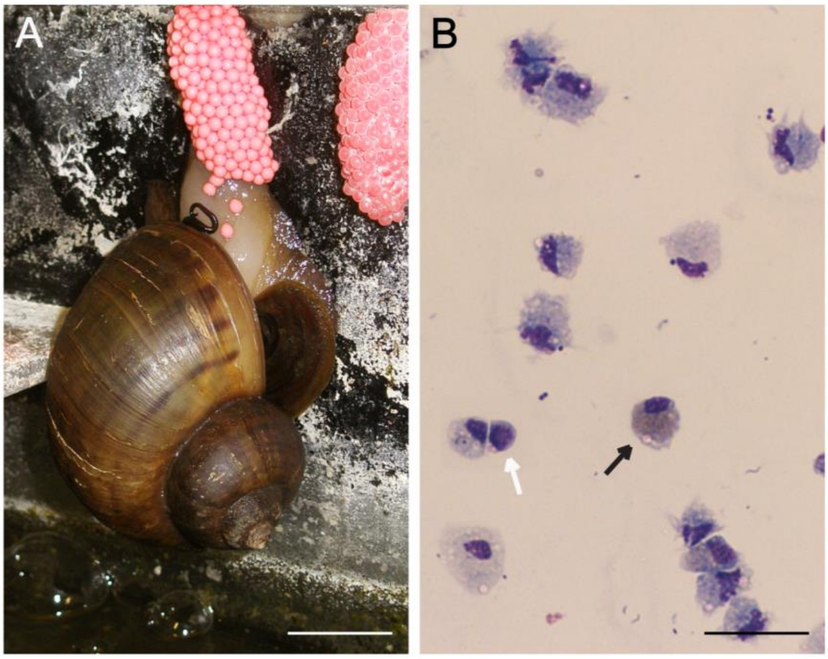

Fig. 1 A) The apple snail *P. canaliculata* laying eggs outside the water onto the wall of the tank. The eggs are bright pink because of the neurotoxic perivitellin fluid contained inside the egg shell. Every egg clutch consists of hundreds of eggs. B) *P. canaliculata* hemocytes. The hemolymph has been collected and cytocentrifuged onto a slide. Hemocytes have been stained with Diff Quik kit. A small blast-like cell (high nuclear/cytoplasmic ratio, white arrow), many large hyalinocytes (agranular blue cytoplasm), and a large granulocyte (granular cytoplasm, black arrows) are present. Bar in A = 2 cm; bar in B = 20 um.

voracious grazer of crops, and this problem is particularly relevant in Asia (Gilioli *et al*., 2017; Lei *et al*., 2017) where this species was introduced as source of food. Unfortunately, *P. canaliculata* was demonstrated to be potentially neurotoxic (Sun *et al.*, 2010) and not edible, and, thanks to the small number of predators, it freely eats and ultimately kills young rice plants (Horgan, 2018; http://www.knowledgebank.irri.org/step-by-step-production/growth/pests-and-diseases/golden-apple-snails). Aware of its impact on the environment and economy, both the EU and some states in the USA implemented tight regulations that forbid circulation and commercialization of the freshwater snails belonging to the genus *Pomacea* (Commission Implementing Decision, 2012; United States Department of Agriculture, Animal and Plant Health Inspection Service [USDA_APHIS]; Lei *et al*., 2017). *Pomacea* and its relationship to the environment have also been studied, suggesting that this snail is a potential bio-indicator as a result of its ability to accumulate heavy metals (Hoang *et al*., 2011).

Apple snails are also interesting from a biomedical perspective because they can regenerate complex organs *de novo*, such as the eyes and the tentacles (important tactile and chemosensory organs). Recent studies have highlighted common aspects and mechanisms shared between adult regeneration and embryonic development (Accorsi *et al*., 2017b). Advances in the understanding of both regeneration and immune responses are especially interesting, and in the past few years, many researchers have been trying to elucidate the deep relationships between regeneration abilities and characteristics of the immune system by comparing different vertebrate and invertebrate model systems with varying regenerative abilities and types of immune systems (Godwin *et al*., 2017; Neves *et al*., 2016; Tasiemski and Salzet, 2017).

The adaptability to different external conditions, the immune-tolerance towards a parasite (Song *et al*., 2016), and the astonishing regenerative capacity of *P. canaliculata* are possible thanks to the role played by a common and fundamental component that is the immune system. As a consequence, the study of the immune system of *P. canaliculata* becomes of wide interest. The characterization of the cellular component of *P. canaliculata* immune system, aka the circulating hemocytes, demonstrated that *P. canaliculata* is not significantly different from other gastropods, since small, blast-like cells (aka pro-hemocytes) and larger hemocytes have been identified (Accorsi *et al.*, 2013; Smith *et al*., 2016). Among the larger hemocytes, hyalinocytes (agranular) and granulocytes (granular cytoplasm) were distinguished, with the former endowed with phagocytic activity (Fig. 1B). Direct observation of the organ structures and the comparison with other gastropod anatomical descriptions allow us to define the pericardial fluid a plausible candidate for the hematopoietic tissue. This tissue has a gel-like texture in younger animals, and it is more fluid in older adults. However, in both cases, it fills the pericardial chamber in which the heart and the ampulla are housed (Accorsi *et al*., 2014). After performing experiments involving repeated hemolymph withdrawal and immunostaining with a mitotic marker, we confirmed the presence of dividing cells in the pericardial fluid, and we hypothesized the involvement of the ampulla as a hemocyte reservoir. The tight functional and anatomical connections between the hemolymph, the heart, the ampulla and the pericardial fluid intrigued us, prompting us to perform further studies on hematopoiesis in the apple snail. Real-time PCR experiments demonstrated the expression of a prokineticin-like protein in the apple snail pericardial fluid, supporting our model of hemocyte replication in this freshwater snail (Accorsi *et al*., 2017a*).* These significant advances represent a solid foundation for studying the dynamic nature of the immune system of mollusks, particularly with respect to hemocyte turnover, maturation, and senescence.

Despite the characterization of hemocytes in mollusks (Smith *et al.,* 2016) and the identification and localization of a hematopoietic tissue/organ (Accorsi *et al*., 2014; Smith *et al.,* 2016), hemocytes maturation still remains to be described, traced, and mechanistically understood. Which anatomical sites are involved in this process? What are the stages of hemocyte differentiation and maturation? When does an hemocyte start to express the receptors that characterize it as a functional immune cell and when it becomes an old and no more functional cell? Can this process be influenced by external events, such as environmental cues or pathogen exposure? (Fig. 2).

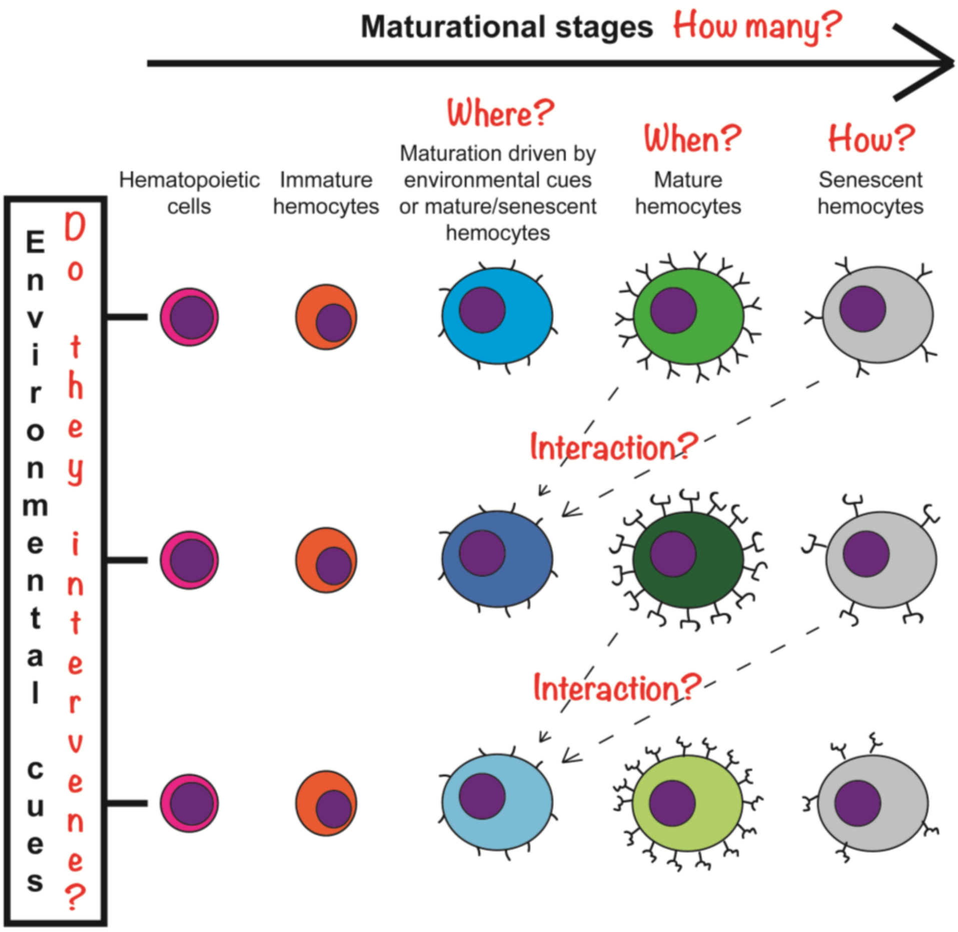

Fig. 2 Schematic representation of the hypothesis described in this paper about the turnover and maturation of *P. canaliculata* hemocytes. The hematopoietic cells (pink cells) proliferate in the hematopoietic organ(s) where they probably both self-renew and give rise to a population of immature hemocytes (orange cells) that differentiate. The differentiating cells mature into the hemocyte stage (green cells), which are functional until they become senescent hemocytes (grey cells) and eventually die. This process has not been carefully dissected in any mollusc. The hypothesis presented here suggests that mature and senescent hemocytes that are already functional can interact with maturing cells (blue cells), influencing their maturation process. This mature/maturing hemocyte interaction would drive the maturation of the new hemocytes towards specific and already encountered targets, enriching for a pool of cells active against the immune challenges that they are likely to face. Besides hemocyte/hemocyte interactions, I hypothesize that also the environmental cues can influence the hematopoietic cell replication rate and hemocyte differentiation process, influencing the compositon of circulating cells on the basis of changes in environmental stimuli. This is, to the best of my knowledge, a new and dynamic perspective for considering the main cellular component of the molluscan immune system, consisting of the circulating hemocytes. The hemocytes have a short lifespan and constant turnover and this might provide to *P. canaliculata* its ability to overcome both persistent and new immune challenges during their long lifespan.

In accordance with observations performed also in other molluscan models (Ottaviani *et al*., 2013; Tascedda *et al.*, 2015; Malagoli and Ottaviani, 2010), the main hypothesis of my laboratory is that the immune system of *P. canaliculata* is constantly refined on the basis of the immune stimulations encountered during an individual organism's lifetime. This would also explain the remarkable capacity of these animals to quickly adapt and begin reproducing in new environments (Accorsi, personal communication; Gilioli *et al*., 2017; Lei *et al*., 2017).

To answer these and many other questions regarding the biology of *P. canaliculata*, several tools are now available thanks to the efforts of several laboratories around the world. Recently, a few organ-specific transcriptomes have been published (Yang *et al*., 2017; Zhou *et al*., 2016), and the Sánchez Alvarado group at the Stowers Institute for Medical Research (Kansas City, MO, USA) is developing an extensive database that includes organ-specific transcriptomes of many adult organs and the transcriptomes of many embryonic stages (Accorsi, personal communication). Proteomic studies have also been performed, which identified the presence of a neurotoxin in the perivitellin fluid of their eggs that likely evolved as defense against predators, expanding the scientific community's interest in this model (Sun *et al*., 2010, 2012; Mu *et al*., 2017). At present, more than one research group is sequencing the genome of *P. canaliculata* in order to obtain a reliable genome database. The first assembly of the genome will soon be published and made available to the community working on apple snails (Accorsi, personal communication; Zhou *et al*., 2016; Guo *et al*., 2018). The availability of these databases will provide our community the opportunity to efficiently search for genes, transcripts, and proteins of interest for both descriptive and functional studies (Malagoli *et al*., 2011; Tascedda *et al.*, 2015; Malagoli *et al.,* 2017). Altogether, this increasing body of evidence and the exponential growth and refining of molecular databases will allow a deeper understanding of the biology of *P. canaliculata* such that a wider set of experiments can be performed.

While the data collected on *P. canaliculata* are increasing, as well as the number of papers published per year on this snail, it is important to emphasize how frequent species misidentification can be among apple snails. In this field, light has been shed by Hayes *et al.* (2009), who described different apple snail species and their phylogenetic relationships, providing a detailed list of approaches to correctly identify each species (Hayes *et al*., 2009, 2015; Guo *et al*., 2018).

The evidence recapitulated in this Vision and Perspectives show that *P. canaliculata* is a versatile model for several studies in the biological field and single it out among mollusks as one of the best candidate to study the process of replication, maturation, and senescence of immune-competent cells in lophotrochozoans.

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