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Title

A prokineticin-like protein responds to immune challenges in the gastropod pest *Pomacea canaliculata*

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Key words

Astakine; prokineticin; hematopoiesis regulation; *Pomacea* transcriptome; molluscan hemocytes; pericardial cavity; gastropod

Highlights

A prokineticin-like protein (*Pc*-PlP) is expressed in several organs of *Pomacea canaliculata*

Pc-plp is maximally expressed in digestive gland, ampulla and hematopoietic tissue

Pc-plp expression is specifically altered in different organs by diverse immune challenges

Pc-plp mRNA appears to be post-transcriptionally regulated as *astakines* in crustaceans

Pc-plp may represent a biosensor to evaluate the immune system status after different challenges

Abstract

The golden apple snail *Pomacea canaliculata* is an invasive pest originating from South America. It has already been retrieved in Asia, the southern United States and more recently in the EU. Aiming to target the immune system of the snail as a way to control its spreading, we have developed organ-specific transcriptomes and looked for molecules controlling replication and differentiation of snail hemocytes. The prokineticin domain-containing protein Astakine 1 is the only cytokine known thus far capable of regulating invertebrate hematopoiesis, and we analyzed the transcriptomes looking for molecules containing a prokineticin domain. We have identified a prokineticin-like protein (PlP), that we called *Pc-plp* and we analyzed by real-time PCR its expression. In control snails, maximum levels of *Pc-plp* were detected in the digestive gland, the ampulla (*i.e.*, a hemocyte reservoir) and the pericardial fluid (*i.e.*, the hematopoietic district). We tested *Pc-plp* expression after triggering hematopoiesis via multiple hemolymph withdrawals, or during bacterial challenge through LPS injection. In both cases a reduction of *Pc-plp* mRNA was observed. The multiple hemolymph withdrawals caused a significant decrease of *Pc-plp* mRNA in pericardial fluid and circulating hemocytes, while the LPS injection promoted the *Pc-plp* mRNA drop in anterior kidney, mantle and gills, organs that may act as immune barrier in molluscs. Our data indicate an important role for prokineticin domain-containing proteins as immunomodulators also in gastropods and their dynamic expression may serve as a biosensor to gauge the effectiveness of immunological interventions aimed at curtailing the spreading of the gastropod pest *P. canaliculata.*

1. Introduction

The freshwater snail *Pomacea canaliculata*, also known as the golden apple snail, is listed as one of the world's worst invasive alien species in the world by the International Union for Conservation of Nature (www.issg.org/worst100_species.html). Originally distributed in South America, *P. canaliculata* is now found in North America, Asia and Europe. In 2012, the EU Parliament (Commission Implementing Decision, 2012) classified *P. canaliculata* as a pest and invading species and in the United States of America strict regulations about management of *P. canaliculata* are already in place [United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA_APHIS)]. Besides representing a threat to crops, *P. canaliculata* is the intermediate host of the nematode *Angiostrongylus cantonensis* responsible for potentially lethal encephalitis (Lv et al., 2009; Song et al., 2016). At present there are no accepted and efficient strategies for controlling the spread of *P. canaliculata*. Snail-specific parasitic nematodes represent one possible solution (Rae et al., 2007). The immunological status of the animals may be crucial in determining the outcome of any treatment with molluscicides of natural origin and, for this, reliable markers of apple snail immune system activation are needed (Accorsi et al. 2013, 2014; Cueto et al., 2015; Song et al., 2014; Xu et al., 2014).

A detailed knowledge of the *P. canaliculata* immune system is indispensable not only if parasitic nematodes are to be used for preventing the spread of the apple snail, but also for identifying potential drugs or compounds able to inhibit hemocyte renewal or maturation. Recently, we have described two circulating hemocyte populations in *P. canaliculata* represented by small (*i.e.*, Group I or blast-like) and large (*i.e.*, Group II) hemocytes. Group II hemocytes display agranular or granular cytoplasm, with the agranular morphology being the most abundant in the hemolymph (Accorsi et al., 2013). Only Group II hemocytes present phagocytic activity towards heat-inactivated *Escherichia coli* (Accorsi et al., 2013).

In adult snails, hemocytes are produced through cell proliferation inside the pericardial cavity, close to the ctenidial-pulmonary and renal veins and within the pericardial fluid, a dense and transparent fluid that fills the pericardial cavity (Accorsi et al., 2014). The ampulla (Accorsi et al., 2014) and the kidney (Cueto et al., 2015) have also been implicated in hemocyte maturation, storage and immune activity. Because the processes of hemocyte generation and maturation shape the immune system and are of primary importance to the immune response, we sought to investigate at a molecular

level the process of hematopoiesis in *P. canaliculata*. The only known cytokine regulating the hematopoietic process in invertebrates is the prokineticin domain-containing protein, Astakine 1, discovered in the crayfish *Pacifastacus leniusculus* (Söderhäll et al., 2005). In crayfish, Astakine 1 promotes the release of new hemocytes from the hematopoietic tissue (Söderhall, 2016). Astakine 1 has also been identified in the tiger shrimp *Paeneus monodon*, and a role in hematopoiesis has also been proposed in this model (Hsiao and Song, 2010). In addition to the crustaceans, prokineticin domain-containing proteins, labeled as astakine-like proteins, have been discovered in other arthropods, *e.g.*, the insect *Lygus lineolaris* (Shelby et al., 2015), and the lophotrocozoan mollusc *Crassostrea gigas,* in which a *Cg*Astakine-like molecule has been shown to increase hemocyte replication rates *in vitro* (Li et al., 2016). In vertebrates, prokineticins are defined as soluble factors involved in several regulatory functions that include immune response (Monnier and Samson, 2008) and neurogenesis (Ayari et al., 2010).

In the apple snail *P. canaliculata,* we identified a divergent prokineticin domain in a sequence that we called, prokineticin-like protein (*Pc*-PlP), by searching for potential astakine-like molecules in transcriptomes generated from various adult organs of this gastropod. We then analyzed the expression of *Pc-plp* and found it to be regulated in an organ-specific fashion after both repeated hemolymph withdrawals and LPS injection. These data suggest that, even though the amino acid sequence is not completely conserved, a role in hematopoiesis and the immune response is likely to be conserved for this molecule.

2. Materials and Methods

2.1. Animals

Pomacea canaliculata specimens were initially purchased from T.A.F. Trans Aquarium Fish srl (Italy) and continuously bred and grown at the Biology Building, Department of Life Sciences, University of Modena and Reggio Emilia (Modena, Italy). *P. canaliculata* were housed in tanks at an approximate density of 1 adult/L. In order to allow egg deposition (which occurs above water level), tanks were filled to half of their capacity with constantly aerated tap water (conductivity 1061 µS/cm at 20°C, hardness 41°F, pH 7.4) (Gruppo Hera, 2015). Two-thirds of th e water were changed twice a week and at the same time frame the animals were fed with different types of green salad. All the animals were maintained at 24 ± 1 °C during bot h routine rearing and experimental

procedures. In order to collect clearer hemolymph and clean tissue/organ samples during the dissection protocol, the animals were kept unfed in separate tanks since 48 h before the experimental starting point up to the end of the experiment. Only adult snails (shell diameter 50 ± 10 mm) were used for the experiments.

2.2. Sequence analysis

A potential astakine was identified by using BLAST algorithm to align bivalve and insect astakines to the *P. canaliculata* transcriptome obtained from the assembly of Illumina high-throughput sequence reads generously shared by Dr. A. Sánchez Alvarado (Stowers Institute for Medical Research, Kansas City, MO, USA) prior to publication. The presence of a predicted prokineticin domains was confirmed using HMMER software (https://www.ebi.ac.uk/Tools/hmmer/) and searching against Pfam.

A comparison between the *P. canaliculata* sequence, invertebrate astakines and vertebrate prokineticin domains was performed through multiple sequence alignments with the online multiple alignment program, MUSCLE, using default parameters (http://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar, 2004). The result was then visualized with the online tool “Multiple align show” (http://www.bioinformatics.org/sms/) that highlights identical (green) and similar (yellow) amino acids with 70% consensus.

2.3. Hemolymph withdrawals and organ dissection

Hemolymph was extracted by applying gentle and continuous pressure onto the disinfected operculum and it was collected with a pipette (Accorsi et al., 2013). About 2 ml of hemolymph were collected from each animal. The samples were then centrifuged at 800 *xg* for 10 min. The supernatant was discarded and the hemocyte pellet suspended in filtered-sterile and RNase-free freshwater snail solution (FSS) (Ottaviani, 1983; Accorsi et al., 2013). The RNA was then extracted from the cell suspension as described in Section 2.6.

After the hemolymph withdrawals the animals were placed in an iced bath for 10 min to anesthetize them before the dissection (Accorsi et al., 2014). Once the shell had been removed, the pericardial fluid was collected with a pipette through a small hole made in the pericardial membrane by micro dissecting scissors. Similarly to vertebrate blood, the pericardial fluid is a fluid tissue constituted by the actively proliferating hemocyte precursors dispersed into a dense liquid (Accorsi et al., 2014). A 50 ± 10 mm adult snail provides between 20 to 25 µl of pericardial fluid. Twenty µl of FSS were used

in order to wash the pericardial cavity and collect the residual pericardial fluid. After pericardial fluid collection, the other organs contained in the pericardial cavity, *i.e.*, heart, ampulla and ctenidial-pulmonary and renal veins (Andrews, 1965, Accorsi et al., 2014) were dissected. Successively, mantle, gills, anterior and posterior kidney, lung and digestive gland (Andrews, 1965) were dissected and collected. Finally, the mouthpart with the peri-esophageal ganglionic ring of nervous system was quickly dissected (Ottaviani et al., 2013) and the pedal ganglia (Ituarte, 1997) were selected and isolated for further analysis. The entire dissection sequence was performed under a dissecting microscope (Wild Heerbrugg AG, Switzerland).

2.4. Repeated hemolymph withdrawals

Repeated hemolymph withdrawals are apparently harmless for the snails but influence the proportion between the different populations of circulating hemocytes. Frequent collections of the differentiated and circulating hemocytes induce the animals to deal with the necessity of introducing new hemocytes in the circulatory system (Accorsi et al., 2014). Hemolymph was collected from seven snails as described in Section 2.3 for 4 times with a 24-h interval between each collection, *i.e*., one hemolymph collection at time 0 and then at 24, 48 and 72 h (Accorsi et al., 2014). All the hemolymph samples collected at different time points were processed for quantitative real time PCR (qPCR) analysis as described in Section 2.6. After the fourth and last hemolymph withdrawal, the snails were dissected and the organs collected following the sequence described in Section 2.3.

2.5. LPS injection

For the immune challenge experiments, animals were divided into two groups of three animals each. The first group of animals was injected with 50 µl of 1 mg/ml FSS-diluted *Escherichia coli*-derived LPS (055:B5) (Sigma-Aldrich, USA) solution (Söderhäll et al., 2005), while the second group (used as a sham-injected control) was injected with 50 µl of FSS (Ottaviani, 1983; Accorsi et al., 2013; Ottaviani et al., 2013). The single injection was performed intramuscularly into the left side of the foot of the animals. The hemolymph was collected as described in the Section 2.3 from each animal 24 h after the injection and then they were sacrificed in order to collect all the organs mentioned in 2.3.

2.6. qPCR

Total RNA was purified from collected organs using Trireagent® (Sigma-Aldrich, USA) and following the manufacturer's protocol. The mRNA was reverse transcribed to cDNA (M-MLV Reverse Transcription System™, Promega, USA) according to manufacturer's instructions. qPCR reactions were then performed using Maxima SYBR Green/Fluorescein qPCR Master Mix (Fermentas, Latvia) with primers amplifying a fragment of the housekeeping gene *ribosomal protein L5* (*rpL5*) and of the target gene *Pc-plp*: *rpL5*_F 5' -CGTATGCCAGAATTGAGGGT- 3', rpL5_R 5' -CAACATCCAAGTATGCACGG- 3', *Pc-plp*_F 5' -ATTGTCTCCGTACTGGCAGC- 3' and *Pc-plp*_R 5' -TCAGCACATCGAAGGAGTTG- 3' following instructions provided by the manufacturer. The applied thermal profile was: 95 °C for 10 min (1 cycle), 95 °C for 15 s and 60 °C for 30 s (40 cycles). All the reactions were performed in triplicate on a CFX96 Touch Real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The analysis was performed using the iQ5 software (Bio-Rad).

2.7. Statistical analysis

Accordingly to Nasi et al. (2015), data are represented as the mean of the ΔCt ± SD, where ΔCt is Ct_{plp} - Ct_{rpL5}, where Ct = threshold cycle. In other words, the higher the ΔCt, the lower the *Pc-plp* mRNA level present in the sample. mRNA variations among different organs and withdrawal time-points were assessed using the non-parametric Kruskal-Wallis test and the Friedman test, respectively. The tissue mRNA changes between control and treated animals were analyzed with non-parametric Mann-Whitney U test. *p* values <0.05 were considered statistically significant.

3. Results

3.1. Sequence similarity

The *Pc-plp* gene sequence identified in our transcriptomes and deposited in GenBank (accession number KY514385) encodes a 121 aa protein (Fig. 1A) that contains a domain characterized by 8 cysteins and some other amino acids with a conserved distribution pattern homologous to the vertebrate domain prokineticin (Fig. 1B). The domain analysis performed on the HMMER website against Pfam revealed the presence of a prokineticin-like domain, with an independent e-value of 0.85 and a conditional e-value of 5.3 e-5, ranging from the 1st to the 102nd aa of the sequence (Pfam prokineticin domain PF06607.9) (Fig. 1C). Moreover, *Pc-plp* shares 38% identity and

53% similarity with a predicted Prokineticin Bm8-f-like of *Crassostrea gigas,* 34% identity and 45% similarity with Astakine-like protein 1 of *Lygus hesperus* and 17% identity and 28% similarity with Astakine 1 of *Pacifastacus leniusculus.* These results would qualify *Pc*-PlP as a potential astakine-like molecule, but the mutation of one nucleotide occurred in our sequence and modified the conserved motif CPC into a CSC tripeptide (Fig. 1B). This led us to consider *Pc*-PlP a divergent prokineticin domain-containing protein and to name it accordingly. We selected the *Pc-plp* sequence for further functional experiment aimed to assess the involvement of this protein in the immune responses of the freshwater snail *P. canaliculata*.

3.2. Pc-plp *distribution in organs*

In control snails, the highest level of mRNA that corresponds to the lower value of average ΔCt, was observed in the digestive gland (ΔCt = -2.14). *Pc-plp* expression was also high in the pericardial fluid, the ampulla and the pedal ganglia (Fig. 2), whose ΔCt were 7.67, 8.54 and 8.68, respectively.

Heart, vessels, anterior kidney, mantle, and gills had similar *Pc-plp* expression, whereas the lowest *Pc-plp* expression level was obtained in the circulating hemocytes (ΔCt = 14.82) (Fig. 2).

3.3. Pc-plp *expression after repeated withdrawals*

Four repeated hemolymph withdrawals in 72 h induced a general decrease *of Pc-plp* mRNA levels (Table 1). The drop of *Pc-plp* mRNA was statistically significant in the pericardial fluid, the circulating hemocytes, and the digestive gland (Table 1). The other organs of the pericardial cavity (ampulla, heart, and vessels), as well as anterior kidney, mantle, gills and pedal ganglia, did not undergo a significant decrease of *Pc-plp* expression (Table 1).

Pc-plp mRNA levels were also evaluated in the circulating hemocytes collected at each withdrawal at 0, 24, 48 and 72 h, respectively. A first significant drop of *Pc-plp* mRNA in circulating hemocytes was observed at the second withdrawal when the average ΔCt is significantly higher than those obtained at 0 h (ΔCt_{0h} = 14.14 and ΔCt_{24h} = 15.33) (Fig. 3). A further significant decrease was observed at the third withdrawal (ΔCt_{48h} = 17.55), while the detected *Pc-plp* mRNA level did not change further in the next and last time point collected (ΔCt_{72h} = 17.18) (Fig. 3).

3.4. Pc-plp *expression after LPS injection*

An overall drop of *Pc-plp* levels was observed also 24 h after 50 mg *E. coli*-derived LPS (055:B5) injection (Table 2). Anterior kidney, mantle, gills and heart presented a significant drop of *Pc-plp* mRNA with respect to sham-injected control snails (Table 2). Among these, the anterior kidney is the organ showing the major reduction of *Pc-plp* mRNA after treatment with LPS, while a milder drop was evidenced in mantle, gills and heart. Also, average ΔCt value of control and treated snails are not significantly different in the other considered organs, including the pericardial fluid, circulating hemocytes and digestive gland (Table 2).

4. Discussion

Circulating hemocytes are key players in cell-based immune response in molluscs (Smith et al., 2016). The study of hemocyte replication, differentiation and the development of their characteristics is crucial to better understand the immune system of *P. canaliculata*, an organism considered to be one of the major invading pests worldwide. With this in mind, transcriptomes of hematopoietic tissue, immune-related components and other organs were generated from adult apple snails and screened for potential hematopoietic factors, such as the prokineticin domain-containing protein Astakine 1 (Söderhall et al., 2005).

Bioinformatic analyses of the *P. canaliculata* assembled transcriptomes indicated the presence of one transcript encoding a 121 aa protein containing a divergent, but still recognizable, prokineticin domain. Thanks to a strictly conserved distribution pattern of 8 cysteines and some other amino acids in crucial positions, important similarities can be detected between *Pc*-PlP and astakine/astakine-like molecules retrieved in arthropods (Söderhäll et al., 2005; Hsiao and Song, 2010; Shelby et al., 2015) and prokineticin domains isolated in several vertebrates (Bullock et al., 2004; Lin et al., 2002). On other hand, the mutation of the highly conserved CPC domain into CSC, led us to call this molecule *Pc-plp*. In mammals, prokineticins have several functions, including the promotion of the myeloid cell lineage differentiation (Dorsch et al., 2005) and angiogenesis in multiple districts (Kurebayashi et al., 2015). The conservation of the prokineticin domain and the evidence published so far in different models suggest an ancient role for these molecules in the proliferation and differentiation of phagocytic immune cells (Söderhäll et al., 2005; Lin and Söderhäll, 2011). A BLAST search of the NCBI NR database reveals homology between *Pc-plp* and a few predicted and

unpublished venom proteins and toxins, such as *Aplysia californica* venom protein 164-like (GenBank accession n. XM_005105364) and U3-aranetoxin-Ce1a-like. This homology confirms the plasticity of the prokineticin domains during evolution and the existence of a family of cysteine-rich secreted proteins that share a certain degree of similarity and have evolved very diverse functions (Fry et al., 2009; Söderhäll et al., 2005). Given that the prokineticin sequence itself is one of the protein scaffold recruited during the evolution into the venoms of various animals (Fry et al., 2009) and that all the venomous molluscs are marine (von Reumont et al., 2014), we hypothesize that *Pc-plp* shares similarities with venom proteins and toxins, but it is not a venom protein itself.

Once identified in *P. canaliculata* transcriptomes, we showed that *Pc-plp* is constitutively expressed in several organs in adults. qPCR experiments demonstrated that in standard condition *Pc-plp* expression is maximal in the digestive gland, the pericardial fluid, the ampulla and the pedal ganglia. The lowest expression level of *Pc-plp* was evidenced in the circulating hemocytes. Our data are consistent with observations made on the prokineticin domain-containing protein, Astakine-like of the bivalve *C. gigas,* where the transcripts of *Cg*Astakine were ubiquitously detected in different tissues (Li et al., 2016). Specifically, the expression of *Cg*Astakine is maximal in the hepatopancreas (*i.e*., digestive gland), and significantly lower in the hemocytes (Li et al., 2016).

P. canaliculata transcriptome analyses of the digestive gland, posterior kidney and lung, also evidenced the presence of bacterial transcriptomes. This is indicative of the presence of commensal microorganisms in *P. canaliculata* organs, an observation already reported in both digestive gland (Castro-Vazquez et al., 2002) and posterior kidney (Cueto et al., 2015) of healthy snails. A close association between bacteria and molluscan tissues in standard conditions is not a rare event, in fact, it has also been observed in healthy oysters, where the average bacterial concentration in the hemolymph has been reported to be 5,7 CFU/mL (Bachère et al., 2015). Our hypothesis supports a conserved role in immunity for the prokineticin-like protein *Pc-plp* and the steady presence of commensal microorganisms into the digestive gland may justify the high expression of this protein in this organ. The high expression of *Pc-plp* in organs related with the generation (the pericardial fluid) and maturation (ampulla) of new hemocytes supports the hypothesis that also in gastropods prokineticin-like proteins may have hematopoietic-related functions.

Other than molluscs, the prokineticin domain-containing astakines and astakine-like molecules have been identified only in arthropods. As in molluscs, arthropod astakines are expressed in several organs. In the insect *L. lineolaris* three astakine-like transcripts are expressed in several tissues and their expression differed between sexes. LlAst-1 and -2 expression was maximal in the fat body (Shelby et al., 2015), an organ that does not have a direct correspondence in molluscan anatomy but that is functionally akin to the molluscan digestive glands (*i.e.*, hepatopancreas) in consideration of its role as nutrient storage organ and metabolic regulator (Dionne, 2014; Tunholi et al., 2016). In crustaceans, the expression of astakine molecules has been principally observed in the hemocytes and hematopoietic tissues (Söderhäll et al., 2005; Hsiao and Song, 2010). In the tiger shrimp, *P. monodon,* the highest expression of PmAst is in the hemocytes but PmAst mRNA has been detected also in other districts such as lymphoid organ, nerve, gills, heart, and hepatopancreas (Hsiao and Song, 2010). This suggests that *Pc*-PIP and the invertebrate astakines, as with vertebrate prokineticin domain-containing proteins, may exert functions beyond a strict hematopoietic modulating cue, like angiogenesis, neurogenesis, immune response and pain perception (Monnier and Samson 2010; Negri and Lattanzi, 2012). We searched for a correlation between immune challenges and *Pc-plp* expression, in order to understand if the prokineticin-like molecule could also represent a good biosensor/marker for the immune system status and activation. Such a biomarker would be of use in developing strategies to control the apple snail spread though the UE and USA by targeting the immune system.

We analyzed *Pc-plp* mRNA response to two independent immune-related challenges. First, we performed repeated hemolymph withdrawals in order to stimulate hematopoietic activity in the apple snail. We have already observed that hemolymph withdrawals cause a change in the population of circulating hemocytes, with a reduction of Group I (blast-like) cells (Accorsi et al., 2014). We observed here that 4 hemolymph the hematopoietic tissue (*i.e.*, the pericardial fluid), circulating hemocytes and digestive gland. The other organs belonging to the pericardial cavity, such as the ampulla, main vessels and heart, appeared to be unaffected. This observation is in agreement with previous experiments demonstrating that none of these organs presented hints of hemocyte replication in previous experiments (Accorsi et al., 2014).

Since in crustaceans the circulating hemocytes appear to be the main regulators of the activity of the hematopoietic tissue (Söderhäll et al., 2005), we investigated *Pc-plp* expression in the collected hemocytes. The time course analysis of *Pc-plp* expression in

the hemocytes revealed a significant drop already at the second collection, and then a second one after the third withdrawal. This change of *Pc-plp* expression may be correlated with the reduction of blast-like cells in the hemocytes population already observed after multiple withdrawals (Accorsi et al., 2014).

As far as the second immune challenge is concerned, also the injection of bacterial extract promoted a general decrease of *Pc-plp* mRNA, but in a different set of organs, namely, anterior kidney, mantle, gills and heart. Specifically, the anterior kidney, the rostral component of an organ containing islets of hemocytes and proposed to be an immune barrier (Cueto et al., 2015), was the most responsive organ, with maximal observed reduction of *Pc-plp* mRNA 24 h after LPS injection. *Pc-plp* mRNA level was also significantly lower than the sham-injected control snails in mantle and gills, two organs in continuous contact with the environment and associated with the molluscan immune system.

The data collected in this study are consistent with those reported in the literature for prokineticin domain-containing proteins of other organisms. In the case of hematopoiesis stimulation, modifications of *Pc-plp* mRNA levels were observed in hematopoietic tissues, that are primarily involved in the regeneration of new hemocytes, as well as in the circulating hemocytes, whose population composition changes with a reduction of blast-like cells. Changes were also observed in the digestive gland, which may be responding to the modified ratio between hemocytes and resident microorganism.

Our data, therefore, suggest that the prokineticin-like protein *Pc*-PIP may have a conserved role in both hematopoiesis process and immune response against a bacterial challenge, here represented by the LPS injection. The potential multiple roles of *Pc*-PIP in hematopoiesis and immune response are supported by the organ-specific responses to the two different stimuli we have applied. Hemolymph withdrawals alter the composition of circulating hemocyte population (Accorsi et al., 2014) and consistently also the mRNA levels of *Pc*-PIP changed in hematopoietic tissue and circulating hemocytes. The immune challenge with bacterial LPS promoted a reduction in the mRNA levels of *Pc-plp* in organs that represent the immune barrier and the first defense of the snails towards the microorganism. Specifically, our results on kidney are in line with the observations of Cueto et al. (2015), which have hypothesized a role as immune barrier for the kidney, where islets of phagocytic hemocytes were observed. In our

experiments, the rostral part of the organ, defined anterior kidney according to Andrews (1965), is the most responsive to the immune challenge by LPS injection.

It might be argued that a decrease in mRNA levels should reflect a decrease in the gene transcription levels. However, as it has been proposed also for astakines and astakine-like molecules in crayfish, shrimps and oyster (Söderhäll et al., 2005; Hsiao and Song, 2010; Li et al., 2016), the drop of *Pc-plp* mRNA likely reflects an increased rate of translation and a consequent decrease of available mRNA, rather than a reduction of the transcription rate. This would indicate that the most responsive organs are not reducing the expression of *Pc-plp* gene, but rather they increase the translation of its mRNA. Further experimentation aimed at quantifying protein levels of Pc-PlP will help resolve this hypothesis.

In sum, we have demonstrated for the first time the presence of a prokineticin-like protein in the gastropod pest *P. canaliculata*, *Pc*-PlP. The expression of *Pc-plp* is altered in an organ-specific fashion by repeated hemolymph collections and LPS injection. This suggests that *Pc*-PlP is involved in both hematopoiesis and immune response of *P. canaliculata* and confirms the functional plasticity of invertebrate prokineticin domains observed also for human and vertebrate prokineticins. Our results represent an important advance for the characterization of hematopoietic process and hemocyte maturation in molluscs and may help in developing strategies for controlling the diffusion of this major invertebrate pest.

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Table 1: *Pc-plp* expression in different organs after 4 hemolymph withdrawals. Control samples are collected from animals that were not previously withdrawn. $\Delta Ct = Ct_{plp} - Ct_{rpL5}$, where Ct = threshold cycle. The organs with a significant change of *Pc-plp* mRNA level after 4 hemolymph withdrawals are marked with a red arrow (n = 5, $p \leq 0.05$).

Tissue	Control (ΔCt ± SD)	Treatment (ΔCt ± SD)	Significant changes
Pericardial fluid	7.67 ± 1.57	11.65 ± 2.19	↓
Ampulla	8.54 ± 2.05	9.60 ± 1.15	None
Hemocytes	14.82 ± 1.09	17.18 ± 2.01	↓
Heart	11.64 ± 0.69	12.31 ± 1.01	None
Vessels	10.84 ± 1.76	11.44 ± 1.29	None
Anterior kidney	11.33 ± 1.37	11.63 ± 1.67	None
Mantle	10.23 ± 2.03	11.88 ± 1.94	None
Gills	10.74 ± 2.56	12.55 ± 0.87	None
Digestive gland	-2.14 ± 1.24	0.60 ± 0.91	↓
Pedal ganglia	8.68 ± 1.38	9.71 ± 1.37	None

Table 2: *Pc-plp* expression in different organs 24 h after LPS injection. Control samples are collected from sham-injected animals. $\Delta Ct = Ct_{plp} - Ct_{rpL5}$, where Ct = threshold cycle. The organs with a significant change of *Pc-plp* mRNA level 24 h after LPS injection are marked with a red arrow (n = 3, $p \leq 0.05$).

Tissue	Control (ΔCt ± SD)	Treatment (ΔCt ± SD)	Significant changes
Pericardial fluid	12.31 ± 1.60	13.45 ± 3.35	None
Ampulla	10.03 ± 2.58	11.77 ± 0.36	None
Hemocytes	14.46 ± 0.96	15.70 ± 1.08	None
Heart	11.79 ± 0.64	12.81 ± 0.34	↓
Vessels	11.34 ± 1.39	11.99 ± 1.89	None
Anterior kidney	6.43 ± 3.85	13.31 ± 0.89	↓
Mantle	8.69 ± 0.59	11.29 ± 0.53	↓
Gills	10.39 ± 1.26	12.59 ± 0.59	↓
Digestive gland	-0.63 ± 2.13	-0.05 ± 1.28	None
Pedal ganglia	10.77 ± 0.87	11.41 ± 0.68	None

Figure legends

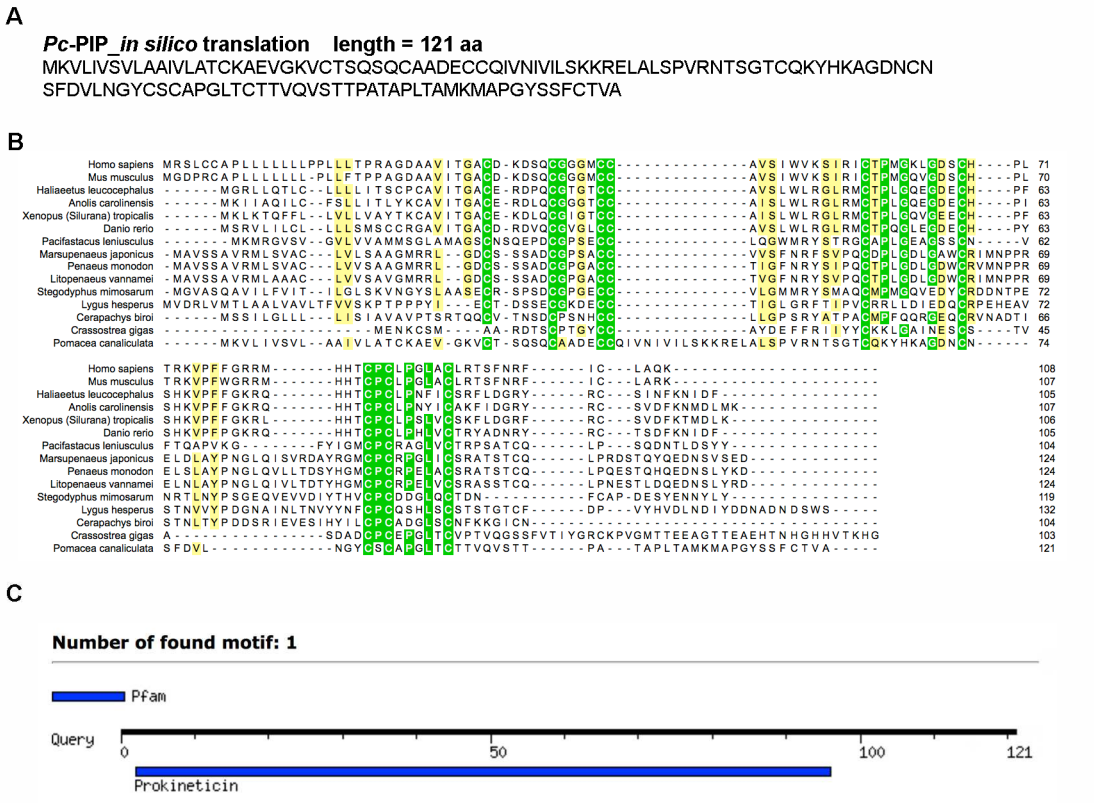

Fig. 1 Bioinformatic analysis of the prokineticin-like sequence identified in the freshwater snail *P. canaliculata.* A) *Pc*-PIP peptide sequence obtained by *in silico* translation. B) Multiple-sequence-alignment of vertebrate prokineticin domains and invertebrate astakines: *Homo sapiens* prokineticin (AAK49919); *Mus musculus* prokineticin (AAM49572); *Haliaeetus leucocephalus* prokineticin (XP_010582900); *Anolis carolinensis* prokineticin (XP_008107764); *Xenopus tropicalis* prokineticin (XP_002933044); *Danio rerio* prokineticin (ACV60345); *Pacifastacus leniusculus* astakine (AAX14635); *Marsupenaeus japonicas* astakine (BAJ34645); *Penaeus monodon* astakine (AAX14636); *Litopenaeus vannamei* astakine (ADM53424); *Stegodyphus mimosarum* astakine (KFM69031); *Lygus hesperus* astakine (JAG12052); *Cerapachys biroi* astakine (EZA54888); *Crassostrea gigas* astakine (EKC19072); *Pomacea canaliculata* (KY514385). A conserved pattern of cysteins and a few other amino acids are highlighted in green (identical amino acids with 70% consensus) and

yellow (similar amino acids with 70% consensus). C) Schematic representation of the prokineticin domain identified in *Pc*-PIP.

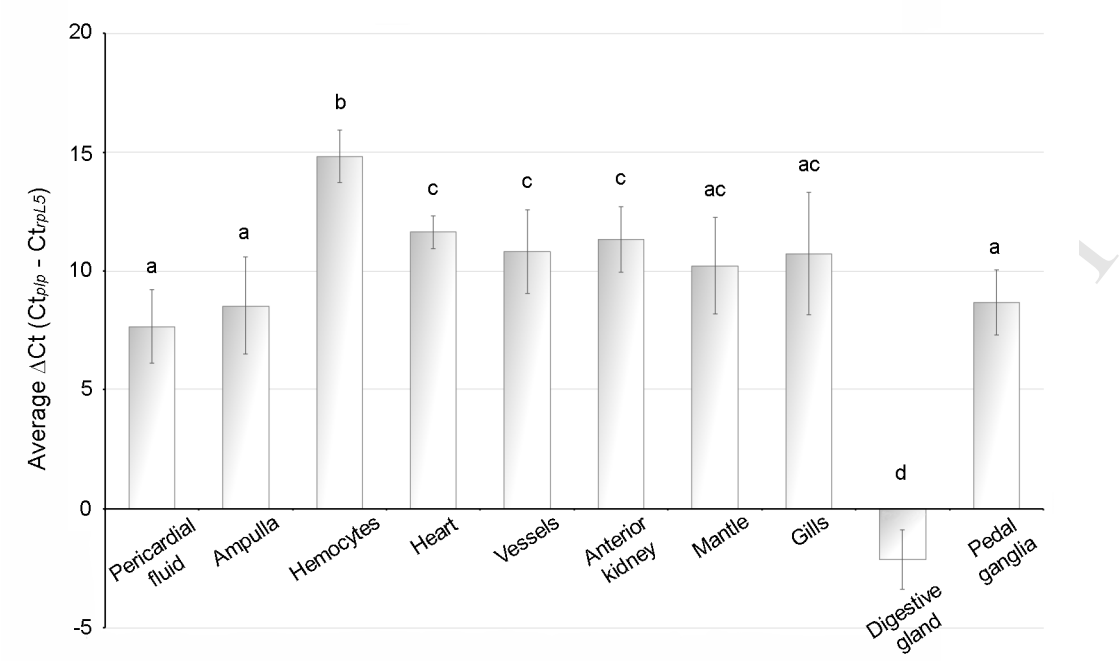

Fig. 2 qPCR for *Pc-plp* performed on several *P. canaliculata* organs. The organs with higher expression of *Pc-plp* in standard condition are the digestive gland, which expression exceeds that of the housekeeping gene *rpl5*, the pericardial fluid (*i.e.*, the hematopoietic tissue), the ampulla, (a hemocyte reservoir) and the pedal ganglia (that belongs to the periesophageal ring of central nervous system) (Ituarte, 1997; Ottaviani et al., 2013). The lowest expression level is observed in the circulating hemocytes. *Pc-plp* expression levels is expressed as ΔCt (n = 5). Different letters indicate significant difference among groups according to Kruskal-Wallis test ($p < 0.05$).

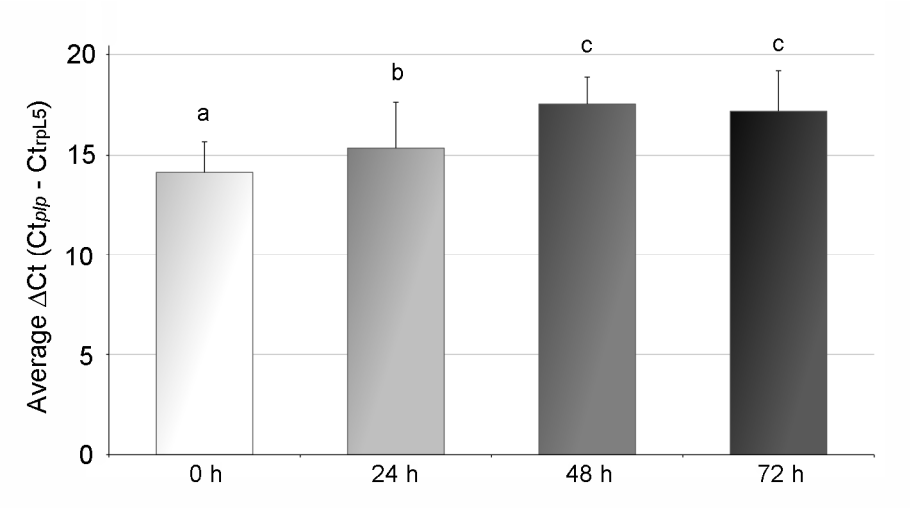

Fig. 3 *Pc-plp* expression in the circulating hemocytes obtained during the first (0 h), the second (24 h), the third (48 h) and the fourth (72 h) hemolymph withdrawals. The expression level of *Pc-plp* was analyzed also in all the hemolymph samples obtained after each withdrawal at 0, 24, 48 and 72 h. A constant reduction of *Pc-plp* expression is observed during time. *Pc-plp* expression levels is expressed as ΔCt (n = 5). Different letters indicate significant difference among groups according to Friedman test ($p < 0.05$).

References

Accorsi, A., Ottaviani, E., Malagoli, D., 2014. Effects of repeated hemolymph withdrawals on the hemocyte populations and hematopoiesis in *Pomacea canaliculata*. Fish Shellfish Immunol. 38, 56-64.

Accorsi, A., Bucci, L., de Eguileor, M., Ottaviani, E., Malagoli, D., 2013. Comparative analysis of circulating hemocytes of the freshwater snail *Pomacea canaliculata*. Fish Shellfish Immunol. 34, 1260-1268.

Andrews, E.B., 1965. The functional anatomy of the mantle cavity, kidney and blood system of some pilid gastropods (Prosobranchia). J. Zool. 146, 70-94.

Ayari, B., El Hachimi, K.H,, Yanicostas, C., Landoulsi, A., Soussi-Yanicostas, N., 2010. Prokineticin 2 expression is associated with neural repair of injured adult zebrafish telencephalon. J Neurotrauma 27, 959-972.

Bachère, E., Rosa, R.D., Schmitt, P., Poirier, A.C., Merou, N., Charrière, G.M., et al., 2015. The new insights into the oyster antimicrobial defense: Cellular, molecular and genetic view. Fish Shellfish Immunol. 46, 50-64.

Bullock, C.M., Li, J.D., Zhou, Q.Y., 2004. Structural determinants required for the bioactivities of prokineticins and identification of prokineticin receptor antagonists. Mol. Pharmacol. 65, 582-588.

Castro-Vazquez, A., Albrecht, E.A., Vega, I.A., Koch, E., Gamarra-Luques, C., 2002. Pigmented corpuscles in the midgut gland of *Pomacea canaliculata* and other Neotropical apple-snails (Prosobranchia, Ampullariidae): a possible symbiotic association. Biocell 26, 101-109.

Cueto, J.A., Rodriguez, C., Vega, I.A., Castro-Vazquez, A., 2015. Immune defenses of the invasive apple snail *Pomacea canaliculata* (Caenogastropoda, Ampullariidae): phagocytic hemocytes in the circulation and the kidney. PLoS One. 10, e0123964.

Commission Implementing Decision., 2012. As regards measures to prevent the introduction into and the spread within the Union of the genus *Pomacea* (Perry), notified under document C. Off. J. Eur. Union 2012:7803 (2012/697/EU), L311/14e15.

Dionne, M., 2014. Immune-metabolic interaction in *Drosophila*. Fly (Austin) 8, 75-79.

Dorsch, M., Qiu, Y., Soler, D., Frank, N., Duong, T., Goodearl, A., 2005. PK1/EG-VEGF induces monocyte differentiation and activation. J. Leukoc. Biol. 78, 426-434.

Edgar, R.C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC bioinformatics 5, 113.

Fry, B.G., Roelants, K., Champagne, D.E., Scheib, H., Tyndall, J.D., et al., 2009. The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. Annu. Rev. Genomics Hum. Genet. 10, 483-511.

Gruppo Hera. Qualità dell'acqua, Media per comune-Dati medi carta dei servizi, Modena-Anno 2015 II semester. http://www.gruppohera.it/gruppo/attivita_servizi/business_acqua/qualita/qualita_acqua_hera/qualita_media_comuni/-modena/pagina178.html

Hsiao, C.Y., Song, Y.L., 2010. A long form of shrimp astakine transcript: molecular cloning, characterization and functional elucidation in promoting hematopoiesis. Fish Shellfish Immunol. 28, 77-86.

Ituarte, C., 1997. *Chilina megastoma* Hylton Scott, 1958 (Pulmonata: Basommatophora): a study on topotypic specimens. Amer. Malac. Bull. 14, 9-15.

Kurebayashi, H., Goi, T., Shimada, M., Tagai, N., Naruse, T., Nakazawa, T., et al., 2015. Prokineticin 2 (PROK2) is an important factor for angiogenesis in colorectal cancer. Oncotarget 6, 26242-26251.

Li, Y., Jiang, S., Li, M., Xin, L., Wang, L., Wang, H., et al., 2016. A cytokine-like factor astakine accelerates the hemocyte production in Pacific oyster *Crassostrea gigas*. Dev. Comp. Immunol. 55, 179-187.

Lin, D.C., Bullock, C.M., Ehlert, F.J., Chen, J.L., Tian, H., Zhou, Q.Y., 2002. Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticin/endocrine gland vascular endothelial growth factor. J. Biol. Chem. 277, 19276-19280.

Lin, X., Söderhäll, I., 2011. Crustacean hematopoiesis and the astakine cytokines. Blood 117, 6417-6424.

Lv, S., Zhang, Y., Liu, H., Zhang, C., Steinmann, P., Zhou, X., et al., 2009. *Angiostrongylus cantonensis*: Morphological and behavioral investigation within the freshwater snail *Pomacea canaliculata*. Parasitol. Res. 104, 1351-1359.

Monnier, J., Samson, M., 2008. Cytokine properties of prokineticins. FEBS J. 275, 4014-4021.

Monnier, J., Samson, M., 2010. Prokineticins in angiogenesis and cancer. Cancer Lett. 296, 144-149.

Nasi, M., De Biasi, S., Bianchini, E., Digaetano, M., Pinti, M., Gibellini, L., et al., 2015. Analysis of inflammasomes and antiviral sensing components reveals decreased

expression of NLRX1 in HIV-positive patients assuming efficient antiretroviral therapy. AIDS 29, 1937-1941.

Negri, L., Lattanzi, R., 2012. Bv8/PK2 and prokineticin receptors: a druggable pronociceptive system. Curr. Opin. Pharmacol. 12, 62-66.

Ottaviani, E., 1983. The blood cells of the freshwater snail *Planorbis corneus* (Gastropoda, Pulmonata). Dev. Comp. Immunol. 7, 209-216.

Ottaviani, E., Accorsi, A., Rigillo, G., Malagoli, D., Blom, J.M., Tascedda, F., 2013. Epigenetic modification in neurons of the mollusc *Pomacea canaliculata* after immune challenge. Brain Res. 1537, 18-26.

Rae, R., Verdun, C., Grewal, P.S., Robertson, J.F., Wilson, M.J., 2007. Biological control of terrestrial molluscs using *Phasmarhabditis hermaphrodita*-progress and prospects. Pest. Manag. Sci. 63, 1153-1164.

Shelby, K.S., Perera, O.P., Snodgrass, G.L., 2015. Expression profiles of astakine-like transcripts in the tarnished plant bug, *Lygus lineolaris*, exposed to fungal spores of *Beauveria bassiana*. Insect Mol. Biol. 24, 480-490.

Smith, V., Accorsi, A., Malagoli, D., 2016. Hematopoiesis and hemocytes in pancrustacean and molluscan models. In: Malagoli D (ed.), The evolution of the immune system. Academic Press 2016: 1-28.

Söderhäll, I., 2016. Crustacean hematopoiesis. Dev. Comp. Immunol. 58, 129-141.

Söderhäll, I., Kim, Y.A., Jiravanichpaisal, P., Lee, S.Y., Söderhäll, K., 2005. An ancient role for a prokineticin domain in invertebrate hematopoiesis. J. Immunol. 174, 6153-6160.

Song H.M., Mu, X.D., Gu, D.E., Luo, D., Yang, Y.X., Xu, M., et al., 2014. Molecular characteristics of the HSP70 gene and its differential expression in female and male golden apple snails (*Pomacea canaliculata*) under temperature stimulation. Cell Stress Chaperones 19, 579-589.

Song, L., Wang, X., Yang, Z., Lv, Z., Wu, Z., 2016. *Angiostrongylus cantonensis* in the vector snails *Pomacea canaliculata* and *Achatina fulica* in China: a meta-analysis. Parasitol. Res. 115, 913-923.

Tunholi, V.M., Tunholi-Alves, V.M., Santos, A.T., Garcia Jda, S., Maldonado, A. Jr, da-Silva, W.S., et al., 2016. Evaluation of the mitochondrial system in the gonad-digestive gland complex of *Biomphalaria glabrata* (Mollusca, Gastropoda) after infection by *Echinostoma paraensei* (Trematoda, Echinostomatidae). J. Invertebr. Pathol. 136, 136-141.

United States Department of Agricuture, Animal and Plant Health Inspection Service (USDA_APHIS). Regulated Organism and Soil Permits, Snails and Slugs. https://www.aphis.usda.gov/aphis/ourfocus/planthealth/import-information/permits/regulated-organism-and-soil-permits/sa_snails_slugs/ct_snails_slugs

von Reumont, B.M., Campbell, L.I., Jenner, R.A., 2014. Quo vadis venomics? A roadmap to neglected venomous invertebrates. Toxins (Basel) 6, 3488-3551.

Xu, Y., Zheng, G., Dong, S., Liu, G., Yu, X., 2014. Molecular cloning, characterization and expression analysis of HSP60, HSP70 and HSP90 in the golden apple snail, *Pomacea canaliculata*. Fish Shellfish Immunol. 41, 643-653.

DCI_16_099 Revised Highlights

A prokineticin-like protein (*Pc*-PlP) is expressed in several organs of *Pomacea canaliculata*

Pc-plp is maximally expressed in digestive gland, ampulla and hematopoietic tissue

Pc-plp expression is specifically altered in different organs by diverse immune challenges

Pc-plp mRNA appears to be post-transcriptionally regulated as *astakines* in crustaceans

Pc-plp may represent a biosensor to evaluate the immune system status after different challenges