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# Upregulated transcription of phenoloxidase genes in the pharynx and endostyle of *Ciona intestinalis* in response to LPS

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ABSTRACT

We investigated the role of phenoloxidases (POs) in ascidians inflammatory reaction, a components of a copper-containing protein family involved in invertebrate immune system. In *Ciona intestinalis* two phenoloxidases (*Cin*PO-1, *Cin*PO-2) have been sequenced. In the present study, real time PCR analysis showed that both *Cin*PO-1 and *Cin*PO-2 genes were modulated by LPS inoculation suggesting that they are inducible and highly expressed in the inflamed pharynx. In situ hybridization disclosed *Cin*PO-1 and *Cin*PO-2 transcripts in pharynx hemocytes (granulocytes) and, mainly, in unilocular refractile granulocytes (URG) which mainly populated the inflamed tunic matrix. Interestingly, the genes are also upregulated by LPS in the endostyle (zones 7, 8 and 9) that is considered homolog to the vertebrate thyroid.



## 1. Introduction

In invertebrates, phenoloxidases (POs) play a key role in melanization, and the "prophenoloxidase activating system" (proPO) comprises an enzyme cascade that leads to active PO and can act as a defensive mechanism in both protostome and deutorostome clades as arthropods, molluscs, annelids, and ascidians (e.g., Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004; Cerenius et al., 2010; Cammarata et al., 1996; Cammarata and Parrinello, 2009; Vizzini et al., 2013). POs are bifunctional copper containing enzymes, components of the arthropod hemocyanin family (Coates and Nairn, 2013), which catalyse both the ortho-hydroxylation of monophenol (i.e., tyrosine) forming o-diphenol, and the dehydrogenation of diphenol into o-quinones which can polymerize producing melanin (Nappi and Seymur, 1991). Tyrosine is converted to (3,4- dihydroxy-l-phenylalanine; DOPA) and the produced DOPA-quinone can form melanin. After hemocytes were stimulated by components of pathogen associated molecular pattern (PAMP), the zymogen is activated via serine proteinases (e.g., Söderhäll and Cerenius,1998; Cerenius and Söderhäll, 2004; Cammarata et al., 1997, 2008; Cerenius and Söderhäll, 2013), and in the arthropods *Pacifastacus leniusculus* these cells degranulate and release inflammatory factors (reviewed in Cerenius et al. (2010)).

In ascidians, a key group in chordate phylogenesis (Swalla et al., 2000; Zeng and Swalla, 2005; Delsuc et al., 2006), POs are involved in inflammation and cytotoxicity against foreign cells or molecules (Akita and Hoshi, 1995; Cammarata et al., 1997, 2008; Ballarin et al., 2005; Hata et al., 1998; Shirae and Saito, 2000; Shirae et al., 2002; Parrinello et al., 2003). The ascidian POs are copper-dependent orthodiphenoloxidases (Kahn, 1985; Sugumaran et al., 1988) that were at first identified by histochemical reaction in the tunic hemocytes (Barrington and Thorpe, 1968), suggesting a quinone-tanning system involved in the production of tunic scleroprotein (Chaga, 1980). Circulating hemocytes from naïve ascidians can exert *in vitro* PO-dependent cytotoxic activity *versus* erythrocytes and tumor cell lines (Cammarata et al., 1997; Arizza et al., 2011), whereas LPS inoculation activates the proPO pathway, and enhanced PO activity (Jackson et al., 1993; Cammarata and Parrinello, 2009). In non-fusion reaction of colonial ascidians, the enzyme mediates the formation of the cytotoxic foci along the contacting regions of genetically incompatible colonies (Hirose et al., 1990; Ballarin et al., 1998; Shirae and Saito, 2000; Shirae et al., 2002; Cima et al., 2004; Zaniolo et al., 2006). And some cells lyse to release the inflammatory factors (Ballarin et al., 2005). In *Ciona intestinalis* an inflammatory response was evoked locally in the tunic matrix and the underlying pharynx by inoculating erythrocytes (Parrinello et al., 1984a,b), foreign proteins (Parrinello, 1981), or lipopolysaccharide (LPS) (Parrinello et al., 2007). Compartment cells mainly populate pharynx vessels of the LPS inoculated ascidians. They express Citype IX-collagen-1α-chain

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(Vizzini et al., 2008), galectin-like lectins (Vizzini et al., 2012), tumor necrosis factor-α-like cytokine (CiTNFα) (Parrinello et al., 2008, 2010), a mannose binding lectin (MBL) (Bonura et al., 2009) and a Cap protein (Bonura et al., 2009, 2010).

The branchial basket consists of two epithelial monolayers where ciliated stigmata are enclosed in a mesh of vessels containing hemolymph (Burighel and Cloney, 1997; Millar, 1953). The ascidian pharynx exerts several physiological role including the immune function (Giacomelli et al., 2012) and suspension feeding. A trough-shaped structure (endostyle) is the first trait of the digestive system, it is a glandular ciliated groove that lays along the ventral wall of the pharynx, it extends to the esophagus, and produces the mucus for suspension feeding. It has been histologically divided in eight functional units called "zones" numbered bilaterally from midventral to dorsolateral (Barrington, 1957, 1958; Fujita and Sawano, 1979; Fujita and Namba, 1971; Thorpe et al., 1972; Ogasawara and Satoh, 1998). In *C. intestinalis*, a long zone 9, consisting of low epithelial cells, further extends outside the endostyle furrow.

In *C. intestinalis* two *Cin*PO genes, *Cin*PO-1 and *Cin*PO-2 (GenBank/EMBL accession numbers AJ547813 *Cin*PO-1 and AJ547814 *Cin*PO-2) have been identified and cloned (Immesberger and Burmester, 2004). The *Cin*-PO1 gene (gene ID 619236) encodes a putative protein of 794 aminoacids (92.0 kDa), the *Cin*-PO2 gene (gene ID 619235) encodes a putative protein of 774 amino acid (86.4 kDa). They show 43.2% sequence identity, lack of a signal peptide indicating a non classical release mechanism, and are clearly distinct from the *C. intestinalis* tyrosinase disclosing a low (12%) identity level between the cDNA sequences (*Cin*TYR, gene ID 251362).

In the present paper, the high proportion of pharynx hemocytes that display PO activity following LPS inoculation appears express *Cin*PO-1 and *Cin*PO-2 as disclosed by real time PCR analysis and in situ hybridization. In addition the *Cin*PO-1 and *Cin*PO-2 transcription result upregulated in cells of endostyle zones, as well as in hemocytes, and tunic cells that populate the inflamed tissues as disclosed by in situ hybridization.

## 2. Material and methods

### 2.1. Ascidians, LPS inoculation and sample preparation

Ascidians were gathered from Termini Imerese marinas (Italy), maintained in aerated sea water, sterilized with UV treatment, at 15 °C and fed every second day with a marine invertebrate diet (Coraliquid, Sera Heinsberg, Germany). LPS (*Escherichia coli* 055:B5, LPS, Sigma–Aldrich, Germany) was prepared in sterile marine solution (MS: 12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 43 mM Tris HCl, 0.4 M NaCl, pH 8.0). According to previous papers, 100 μg LPS in 100 μl MS *per* specimen were inoculated into the median region of the body wall just under the tunic. Only animals with clear tunic, without epiphytic formations or injury were used. Ascidians, either untreated (naïve) or injected with 100 μl MS, were used as a control. A suitable amount (200 mg/ascidian) of pharynx tissue was excised at various time-points p.i. (1–48 h) from the injection region of the body wall, immediately soaked in RNAlater Tissue collection (Ambion, Austin, TX), and stored at −80 °C or fixed for histology. Hemocyte types (lymphocyte like cell; pigment cell; hyaline amoebocyte; granulocyte with large granules amoebocyte; univacuolar refractile granulocyte (URG), with a single prominent refractile inclusion in a large vacuole that nearly filled the whole cell; signet-ring cell, similar to a URG with nonrefractile material in the vacuole) were identified according to Cammarata et al. (1993) and Arizza and Parrinello (2009).

### 2.2. Pharynx total RNA extraction, cDNA synthesis and real-time PCR analysis

Total RNA was isolated from the pharynx tissue by using an RNAqueous™-Midi Kit purification system (Ambion) and reverse-transcribed by the Cloned AMV First-Strand cDNA Synthesis Kit.

Tissue expression of the *Cin*PO-1 and *Cin*PO-2 genes were examined by real-time PCR analysis with the Sybr-Green method (Applied Biosystems 7500 real-time PCR system), as previously described. Primers were designed by using Custom Primers OligoPerfect Designers software (https://tools.invitrogen.com/) and synthesized commercially (Eurofins MWG Operon, Ebersberg, Germany). Tissue expression was performed in a 25-μl PCR containing 2 μl cDNA converted from 250 ng total RNA, 300 nM *Cin*PO-1 forward (5′-ATACCCGGACAAGATCACCATG-3′) and *Cin*PO-1 reverse primers (5′-TGGAGAGGTTCTCAGCTGCTTC-3′), 300 nM *Cin*PO-2 forward (5′-CCCCTATTAGAGTGAATGGCCA-3′) and *Cin*PO-2 reverse primers (5′-CAAAGAGATCCACTGGTGCAGA-3′), 300 nM actin (Accession Number AJ297725) forward (5′-TGATGTTGCCGCACTCGTA-3′) and actin reverse (5′-TCGACAATGGATCCGGT-3′) primers, and 12.5 μl Power Sybr-Green PCR Master Mix (Applied Biosystems). The 50 cycles of the two-step PCR program consisted of initial polymerase activation for 3 min at 95 °C followed by a denaturing step at 95 °C for 15 s, and then annealing/extension was carried out at 60 °C for 45 s when the fluorescent signal was detected. Each set of samples was run three times, and each plate contained quadruplicate cDNA samples and negative controls. The specificity of amplification was tested by real-time PCR melting analysis. To obtain sample quantification, the $2^{-\Delta\Delta Ct}$ method was used, and the relative changes in gene expression were analyzed as described in the Applied Biosystems Use Bulletin N.2 (P/N 4303859). The amount of *Cin*PO-1 and *Cin*PO-2 transcript from the various times were normalized to actin in order to compensate for variations in input RNA amounts. Relative *Cin*PO-1 and *Cin*PO-2 expression were determined by dividing the normalized value of the target gene in each time by the normalized value obtained from the untreated tissue.

### 2.3. Histological methods

Pharynx fragments containing the endostyle or fragments with tunic and pharynx tissues, were excised from the ascidians. Tissues were fixed in Bouin's fluid (saturated picric acid:formaldehyde:acetic acid, 15:5:1) for 24 h, paraffin-embedded and serially cut into 6 μm sections (Leica RM2035 microtome, Solms, Germany). Histological sections were examined under a Leica DMRE microscope. Body wall tissues and cells were identified as reported previously (Vizzini et al., 2008; Parrinello et al., 2008).

### 2.4. In situ hybridization assay (ISH)

To examine tissue excised from the inflamed body wall, ISH was carried out with digoxigenin-11-UTP-labeled riboprobes (1 μg/ml final concentration). The *Cin*PO-1 probe was generated by PCR amplifying a cDNA fragment of 350 bp of cDNA using the *Cin*PO-1 forward primer (5′-AAACACTTTGAGGGAGAAAT-3′) and the *Cin*PO-1 reverse primer (5′-ATGACATACAAGGATCAAC-3′). The *Cin*PO-2 probe was generated by PCR amplifying a cDNA fragment of 270 bp of the cDNA using the *Cin*PO-2 forward primer (5′-AATAGAAAATGCGATGTCTG-3′) and the *Cin*PO-2 reverse primer (5′-GGATAGTGTTGAAGTTGGTG-3′). The DNA fragments were cloned in the pCR4-TOPO vector (Invitrogen, USA).

The digoxigenin-11-UTP-labeled riboprobes was carried out according to manufacturer's instructions (Roche Diagnostics). The re-hydrated histological sections were digested with proteinase K (10 μg/ml) in PBS for 5 min, washed with PBS-T, and treated for

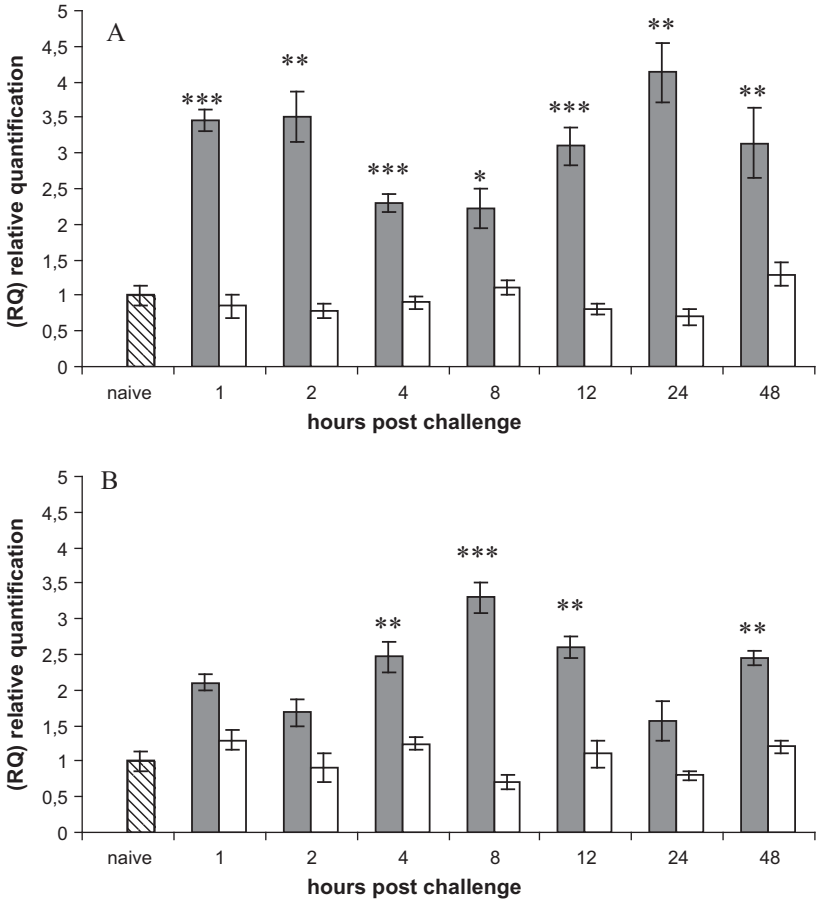


**Fig. 1.** Real-time PCR analysis. Time-course of *Cin*PO-1 (A) and *Cin*PO-2 (B) gene expression in *Ciona intestinalis* pharynx after inoculation into the body wall of 100 μg bacterial lipopolysaccharide (LPS, grey column) in 100 μl marine solution (MS, white column), compared with the gene expression in ascidians injected with 100 μl MS. Values, plotted as mean ± SD, were inferred from four ascidians examined in three distinct experiments; each assay was performed in triplicate. Significance was evaluated by comparing the values with the expression level of untreated pharynx from four naïve ascidians. $^{**}P < 0.01$, $^{***}P < 0.001$.

hybridization with 50% formamide, 5× SSC (1 × SSC: 0.15 M NaCl/0.015 M sodium citrate, pH 7), 50 μg/ml heparin, 500 μg/ml yeast tRNA, and 0.1% Tween 20, at 37 °C overnight. After washing in PBS-T and 4XSSC (twice for 10 min), the sections were incubated for 1 h with anti-DIG-Fab-AP conjugate (Roche Diagnostics) diluted 1:500 and washed in PBS-T. Finally, the sections were incubated in the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma–Aldrich, Germany). Color development was stopped after 30 min at room temperature.

### 2.5. Statistical analysis

Student's *t*-test was used to estimate statistical significance. Multiple comparisons were performed with one-way analysis of variance (ANOVA), and different groups were compared by using Tukey's *t*-test. Standard deviations were calculated on four experiments. $P < 0.01$ was considered statistically significant.

## 3. Results

### 3.1. Pharynx CinPO-1 and CinPO-2 gene expression is differently upregulated by LPS

To study the expression pattern of the *Cin*PO-1 and *Cin*PO-2 genes, quantitative mRNA expression of *Cin*PO-1 and *Cin*PO-2 in naïve, MS inoculated ascidians and LPS inoculated ascidians, were checked by Real Time PCR analysis. Four ascidians in three distinct experiments were examined at different post-inoculation time points (1, 4, 8, 12, 24, 48 h p.i. The LPS inoculation significantly enhanced the *Cin*PO-1 and *Cin*PO-2 expression levels (Fig. 1). In particular, the *Cin*PO-1 (Fig. 1A) mRNA level, also compared to *Cin*PO-2, was enhanced at 1 h p.i. ($P < 0.001$), decreased at 4–8 h, and reached the maximum at 24 h p.i. ($P < 0.01$). Conversely, the *Cin*PO-2 (Fig. 1B) expression was enhanced at 4 h p.i. ($P < 0.01$) reaching the highest expression at 8 h p.i. ($P < 0.001$), then decreased, and low levels were found at 12–24 h p.i. ($P < 0.01$), finally a small increase was again found at 48 h p.i. ($P < 0.01$). The response by MS inoculated ascidians indicated that the inoculation procedure *per se* did not significantly upregulated the *Cin*PO-1 and *Cin*PO-2 genes.

### 3.2. Pharynx and tunic hemocytes are marked by CinPO-1 and CinPO-2 riboprobes

Fig. 2 shows ISH assay of pharynx histological sections from MS-inoculated ascidians (Fig. 2A, E, I, O, Q) and LPS-inoculated ascidians examined with *Cin*PO-1 riboprobe at 8 h p.i. (Fig. 2L–N, P, R) and with *Cin*PO-2 at 24 h p.i. (Fig. 2B–D, F–H). The time points after LPS inoculation were chosen in accordance to the peaks of gene expression showed by the real time PCR analysis. *Cin*PO-1 (Fig. 2A and E) and *Cin*PO-2 (Fig. 2I, O, Q) riboprobe marked cells can be found in vessels of MS-inoculated ascidians. At 8 h after LPS inoculation, although a cell count was not carried out, a large part of the pharynx vessels appeared to be densely populated with hemocytes marked by the *Cin*PO-1 (Fig. 2B and F) and *Cin*PO-2

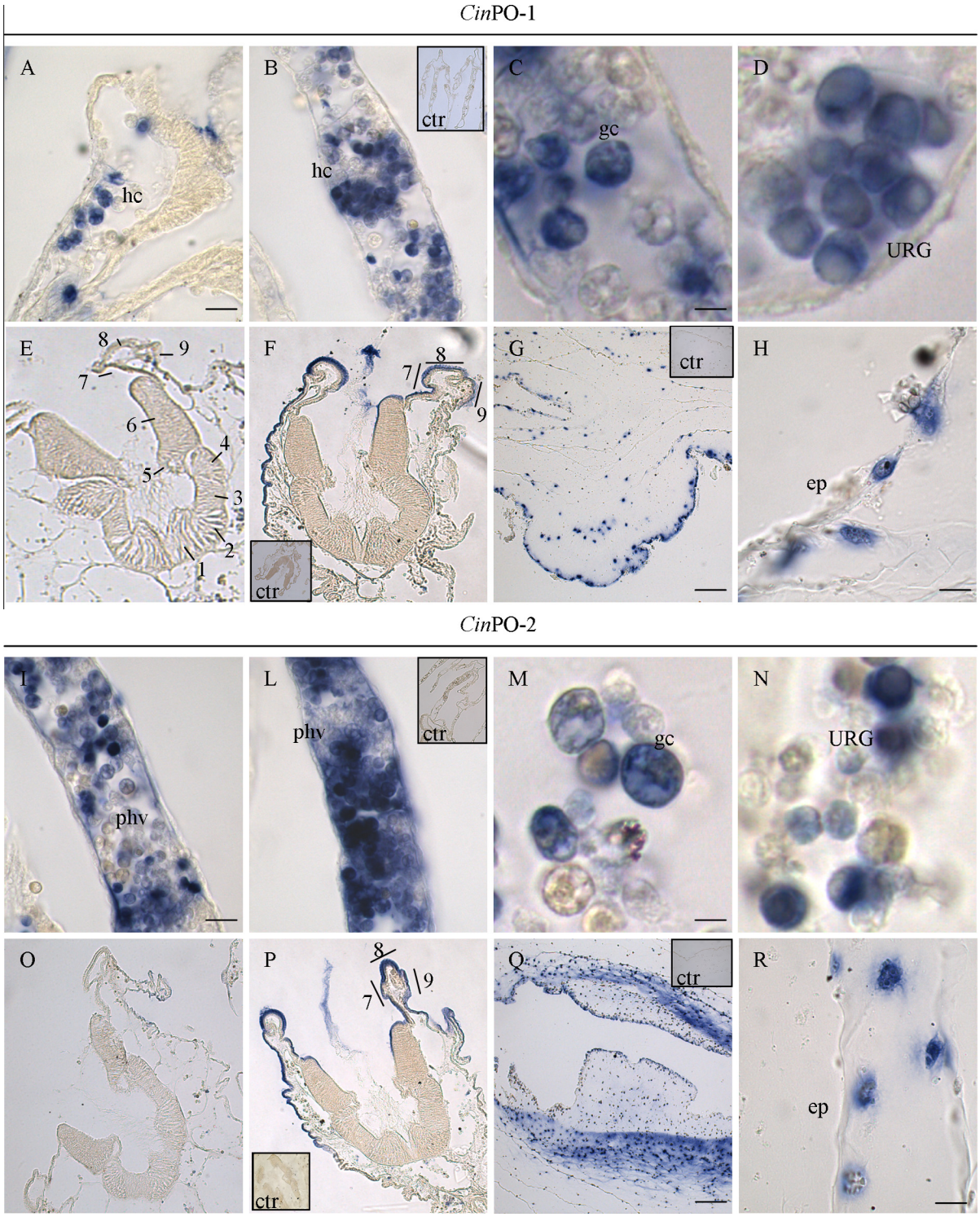


**Fig. 2.** Histological sections of *Ciona intestinalis* pharynx. In situ hybridization with the *Cin*PO-1 riboprobe: MS-inoculated ascidian (A and E) and ascidian at 24 h after LPS challenge (B–D, F–H). In situ hybridization with the *Cin*PO-2 riboprobe: MS-inoculated ascidian (I, O, Q,) and ascidian at 8 h after LPS inoculation (L–N, P, R). Vessels (A–D, I–N); endostyle (E, F, O, P); tunic (G, H, Q, R). Bars size: 40 μm (A, B, G–I, L, O, P); 100 μm (E, F, Q, R); 10 μm (C, D, M, N), 5 μm (insets). phv: pharynx vessels, hc: hemocyte cluster, ep: epidermis, URG: univacuolar refractile granulocyte, gc: granular cells, ctr: control.

(Fig. 2L) riboprobes. Among the various hemocyte types, both the transcripts were found in granulocytes with large granules and URG characterized by a unique large granule that occupies the cytoplasm (Fig. 2C, D, M, N). The signal was found in the cytoplasm rim around the granules.

The granulocyte and URG population density also increased in the tunic close to the pharynx, and most cells were marked by both riboprobes (Fig. 2G, Q, R).

The epidermis under the tunic was negative, whereas granulocytes that express the *Cin*POs were spread within the lacunae lin-

ing the epidermis (Fig. 2H). Histological sections treated with the sense strand did not display any positive staining.

### 3.3. LPS upregulates CinPO-1 and CinPO-2 genes in endostyle cells

Fig. 2 (E and O) shows pharynx sections disclosing the endostyle. The bottom of the groove (zone 1) is lined with a longitudinal row of very long cilia that move the mucus. The zone 2 is formed by elongated columnar cells arranged more or less parallel with each other; the cells of the median zone 4 resembles the histological organization of the ventral zone 2; the zone 3 presents markedly narrow ciliated cells; the zone 5 consists of very low and narrow epithelial cells; the zone 6 is the largest area in the transverse sections, the apical surface of cells discloses cilia.

Both *Cin*PO-1 and *Cin*PO-2 riboprobes marked the cells of zones 7, 8 and 9 (Fig. 2F and P), these are longer zones consisting of low epithelial cells (3–6 μm in height and 4–10 μm in width). The cells of both zones are similar in their features, but the cells of the zone 8 are ciliated. Fig. 2(F and P) shows that the zone 9, consisting of low epithelial cells, extends for a length outside the groove.

ISH assay disclosed that LPS inoculation upregulated the *Cin*PO-1 (Fig. 2F) and *Cin*PO-2 (Fig. 2P) genes in the zones 7, 8 and 9 of the endostyle. No signals were found in endostyle sections from MS-inoculated ascidians (Fig. 2E and O).

## 4. Discussion

As shown by Cammarata et al. (2008), hemocytes containing POs are involved in the *C. intestinalis* inflammatory response to LPS. A dopa-MBTH assay of the tunic homogenate supernatant showed an enhanced $Ca^{2+}$-independent PO activity (Cammarata et al., 2008). The inoculated LPS permeates both tunic and pharynx tissues stimulating the expression of several immune-related factors (Parrinello et al., 2007, 2008, 2010; Vizzini et al., 2008; Cammarata et al., 2008; Bonura et al., 2009, 2010; Cammarata and Parrinello, 2009; Vizzini et al., 2012), and hemocytes, most granulocytes and URGs, are recruited into the tissues and were positive for PO activity (Parrinello, 1981; Parrinello et al., 1984; Parrinello and Patricolo, 1984). In the pharynx, the real time PCR analysis showed that *Cin*PO-1 gene expression peaked at 2 and 24 h, and *Cin*PO-2 gene expression reached the peak at 8 h p.i. The entire outline of the inflammatory response, as the sum of both *Cin*PO genes expressions, showed an increase of these genes, the upregulation started quickly following LPS inoculation and was found up to 24 h.

In situ hybridization with *Cin*PO-1 and *Cin*PO-2 riboprobes disclosed the expression of both *Cin*PO-1 and *Cin*PO-2 genes by inflammatory granulocytes and URGs. A basal expression of both genes was found in naïve and MS-inoculated ascidians, indicating an active role of the enzymes as a constitutive defense activity as befits innate type defense factors. However, following LPS inoculation, a large part of the pharynx vessels, positive URGs were numerous in the tunic matrix close to the pharynx suggesting a challenge of tunic cells, and cell recruitment from the pharynx. The increased number of cells that expressed the *Cin*PO-1 and *Cin*PO-2 immune-related genes supported the role of the pharynx as the main organ of the ascidian immune system. Although the higher level of specific mRNAs revealed by the PCR analysis could be imputable to the increased number of inflammatory hemocytes, the in situ hybridization assay showed that the largest part of them were active cells in which the *Cin*PO-1 and *Cin*PO-2 genes were upregulated. The possibility exists that the increase in number of PO-expressing cells could be due to enhanced proliferating activity of hematopoietic tissue in the pharynx (Ermak, 1976) stimulated by the LPS challenge.

Interestingly, in situ hybridization assay showed that the enhanced *Cin*PO-1 and *Cin*-PO-2 genes expression by the cells of the endostylar zones 7, 8 and 9, supporting the LPS effect on the transcription upregulation of these genes. The endostyle has several roles including suspension feeding and secretory function. According to previous reports (Olsson, 1963; Fujita and Namba, 1971; Thorpe et al., 1972; Ogasawara and Satoh, 1998; Ogasawara et al., 1999a,b; Ogasawara, 2000; Ristoratore et al., 1999; Venkatesh et al., 1999), the zones 1, 3 and 5 are supporting elements involved in catching and transporting food, the zones 1–4 produce the mucus, a complex of mucoproteins and mucopolysaccharides that can capture particles as small as bacteria (reviewed in Petersen (2007)), the zones 2 and 4 produce galectins following LPS inoculation (Parrinello et al., 2015), and the zones 7, 8 and 9 are thyroid-equivalent elements that incorporates iodine in iodoproteins, and have been retained homolog to the vertebrate thyroid (Ogasawara and Satoh, 1998; Ogasawara et al., 1999a, 1999b; Ogasawara, 2000; Ristoratore et al., 1999; Venkatesh et al., 1999). Recently, in the zones 7–9, the expression of a cell-adhesive peroxinectin-like that may be involved in the proPO system, has also been shown (Vizzini et al., 2013). Although we have only preliminary results, the colocalization of the two enzyme in hemocytes and endostyle and a possible cooperative function to produce cytotoxic molecules justify a future functional study. Albeit a relation among POs, *Cin*Pxt and peroxidase activity, expressed in these zones could be hypothesized, the present data on PO expression do not allow to establish a functional relationship between POs gene expression and thyroid-like activity during the inflammatory response.

However, a recent paper reported that human thyroid cells express functional sensors for exogenous and endogenous dangers launching innate immune responses without the assistance of immune cells (Kawashima et al., 2013).

Our results provide compelling evidence of a complex involvement of the whole pharynx, including the endostyle, in the inflammatory response to LPS. Interestingly, POs could be challenged by filtered bacteria, and the *Cin*PO genes transcription can be modulated to exert a defense role.

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