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RESEARCH REPORT

Amyloid/Melanin distinctive mark in invertebrate immunity

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Abstract

Protostomes and Deuterostomes show the same nexus between melanin production, and amyloid fibril production, *i.e.*, the presence of melanin is indissolubly linked to amyloid scaffold that, in turn, is conditioned by the redox status/cytoplasmic pH modification, pro-protein cleavage presence, adrenocorticotropin hormone (ACTH), melanocyte-stimulating hormone (α-MSH), and neutral endopeptidase (NEP) overexpressions. These events represent the crucial component of immune response in invertebrates, while in vertebrates these series of occurrences could be interpreted as a modest and very restricted innate immune response. On the whole, it emerges that the mechanisms involving amyloid fibrils/pigment synthesis in phylogenetically distant metazoan (*viz,* cnidaria, molluscs, annelids, insects, ascidians and vertebrates) are evolutionary conserved. Furthermore, our data show the relationship between immune and neuroendocrine systems in amyloid/melanin synthesis. Indeed the process is closely associated to ACTH-α-MSH production, and their role in stress responses leading to pigment production reflects and confirms again their ancient phylogeny.

Key Words: amyloid fibrils; melanin; ACTH, α-MSH; neutral endopeptidase; invertebrate immunity

Introduction

Living organisms produce melanin as defense system against attack, harm, or injury coming from any type of non-self (Golkar *et al*., 1993; Nappi and Ottaviani, 2000; Petes *et al*., 2003; Nappi, 2010). In vertebrates, it is well known that the biopigment shows its main quality neutralizing the potentially deleterious effects of sunlight (Edelstein, 1977). Melanin production is considered a widespread event, and becomes essential in those invertebrates where invaders such as parasites or fungi, are rapidly isolated and sequestered in a capsule made of pigment and hemocytes (Carton *et al*., 2008). In general melanin, acting as scavenger of reactive oxygen species (ROS), defends cells/tissues from the toxic effects of free radicals and it is manifested, from invertebrates up to man, in the areas of tissue repair, during regeneration process and in response to pathogens (de Eguileor *et al.* 2000; Gourdon *et al*., 2001; Ballarin *et al.*, 2002; Nappi and Christensen, 2005; Lewis and Pollard, 2006; Nappi, 2010; Palmer *et al*., 2011). Melanin biosynthesis is due to the activation of prophenol oxidase (pro-PO) system present in cell and/or in body fluids. The pro-PO activating system is best understood in crayfish *Pacifasticus leniusculus* (Söderhäll and Smith, 1986), in silkworm *Bombyx mori* (Ashida and Yoshida, 1990; Yasuhara *et al*., 1995), in *Drosophila melanogaster* (Nappi and Vass, 1993; Fujimoto *et al*., 1995), in Echinoderms such as *Holoturia tubulosa* (Roch *et al.*, 1992), in Ascidians (Jhoanson and Söderhäll, 1989; Cammarata and Parrinello, 2009; Ballarin, 2012), and in Cephalochordates (Pang *et al*., 2004). Specifically about biopigment synthesis, in Cnidaria several papers show that pathogens or any kind of stressors, induces a localized melanization in sea fan corals. The pigment production is due to an augment of amebocyte melanosome production and to pro-PO activity in the tissues (Petes *et al*., 2003; Mydlarz *et al*., 2008). In Annelida (oligochaets and polychaets), several authors (Porchet-Henneret, 1987; Valembois *et al*., 1988; Porchet-Henneret and Vernet, 1992; Beschin *et al*., 1998; Fyffe *et al*., 1999; Adamowicz, 2005; Prochazkova *et al*., 2006) describe the efficient activation of both pro-PO cascade in coelomic fluid and in a subpopulation of granulocytes, with the final

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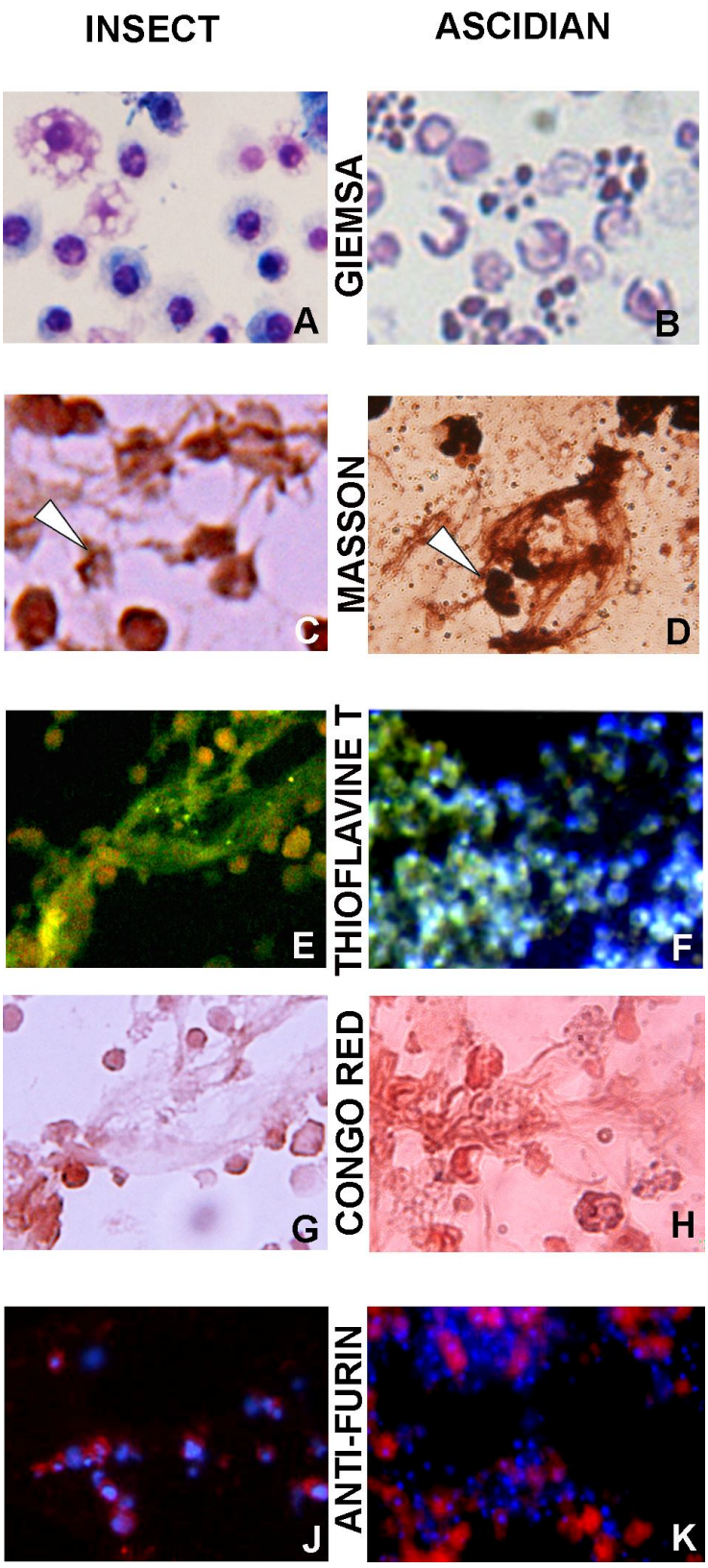

Fig. 1 Circulating hemocytes from insect and ascidian. (A-B) Light microscopy: May Grunwald Giemsa technique (the circulating cell cytoplasm is differently stained in relation to their cytoplasmic pH) (pink mark indicates acid pH); (C-D) Light microscopy: Masson Fontana technique: silver staining demonstrates melanin deposition (arrowheads); (E-H) Fluorescence and light microscopy: thioflavine T (E-F) and Congo red (G-H) staining recognize amyloid or amyloid-like structures; (J-K) Fluorescence microscopy: Immunocytochemical evidence of furin-like protein (in red), nuclei in blue are stained with DAPI.

result of massive melanin production. An extensive literature is reported about humoral factors and cellular responses in molluscs, against any type of non-self (Ottaviani and Cossarizza, 1990; Ottaviani, 2006; Novoa *et al*., 2011). In particular, a classification of hemocytes has been proposed according to their different function in immune responses with particular regard to melanin synthesis (Ottaviani, 1983; Ottaviani and Franchini, 1988; Ottaviani *et al*., 1990, 1993; Matricon-Gondran and Letocart, 1999; Gorbushin and Iakovleva, 2006; Jiravanichpaisal *et al*., 2006; Koropatnick *et al*., 2007; Mahilini and Rajendran, 2008; Venier *et al*., 2011). Moreover, several authors have described in mollusc bivalves and gastropod granulocytes, involved in wound repair or internal defense, the presence of cytoplasm membrane-limited granules containing "filamentous matrix" with acid phosphatase activity of unknown function (Giamberini *et al.*, 1996; Matricon-Gondran and Letocart, 1999). Induced melanization/encapsulation against non-self is well known also in arthropods (Söderhäll and Smith, 1986; Ashida *et al*., 1990; Hoffman and Reichart, 2002; Martin *et al*., 2007; Gallo *et al.,* 2011). With reference to insects, two cell types are responsible for the entrapment of the invaders and this process is generally accompanied by the phenoloxidase activity inducing the formation of the melanotic material. These events have been well-studied in insect host/parasitoid model (*Heliothis virescens*/*Toxoneuron nigriceps*) (Ferrarese *et al*., 2005; Falabella *et al.,* 2012; Grimaldi *et al*., 2012).

The same implications are also evident in Deuterostomes such as Echinoderms (sea urchin, holoturians) (Canicatti and Seymour, 1991) and Tunicates (sea squirts) (Shirae *et al*. 2002; Hirose, 2003; Ballarin, 2008; Cammarata and Parrinello, 2009; Ballarin, 2012). In Tunicates, morula cells are able to recognize the presence of foreign elements and release phenoloxidase which induces melanin formation (Hirose, 2003; Ballarin *et al.,* 2005; Ballarin, 2012). In mammals, melanocytes are cells with the main function in synthesizing and packaging the brown pigments in melanosomes to protect the skin against ultraviolet radiation (UV). As previously mentioned, we have demonstrated that in the insect *H. virescens* larvae, during the earliest phase of the parasitization, melanin was packaged due to the production of large amount of amyloid fibrils, sharing these linked events with vertebrates, where, as suggested by Fowler and coworkers (2006), amyloid fibrils template and accelerate the formation of pigment. The principal divergence in melanization process of insects and vertebrates is that it takes place in specific cell types (granulocytes and melanocytes, respectively), but in insect cells the phenomenon is faint in respect to that observed in the hemocel, where large amount of pigment are derived from sieric pro-PO system reactions. On the contrary, in vertebrates, the massive melanin synthesis occurs intracellularly, *i.e.,* in melanosomal organelles (Grimaldi *et al.,* 2012).

On the basis of our previous data (Falabella *et al*., 2012; Grimaldi *et al*., 2012) and the evidence (previously mentioned), we surmize that protostomes and deuterostomes, show the same nexus between melanin production, and amyloid fibril production, *i.e.*, the presence of melanin is indissolubly linked to amyloid scaffold that, in turn, is due to a combined redox status/cytoplasmic pH modification, pro-protein cleavage presence, adrenocorticotropin hormone (ACTH), melanocyte-stimulating hormone (α-MSH), and neutral endopeptidase (NEP) overexpressions. Thus, in the present paper, using a variety of techniques we confirm our hypothesis.

Materials and Methods

Hemocytes extraction and culture

Hemocytes from several species stimulated with LPS injection (*Helix pomatia, Heliothis virescens, Ciona intestinalis*) were collected by centrifuging the circulating fluid at 400 g per 7 min at 4 °C. The pellet washed with MEAD-PBS solution (1:1). The hemocytes were resuspended in complete medium (Grace's medium, FBS 10 %, antibiotic-antimicotic solution 1 %, SIGMA) and were plated at concentration of $1x10^6$ cells/ml into 24-well culture plates. The B16-F10 murine melanoma cell line (derived from C57BL/6J mouse, *D/D*) was a generous gift from Prof. Douglas Noonan (University of Insubria, VA, Italy). B16-F10 murine melanoma cells were cultured for 24 h in DMEM and 10 % FBS and then changed to DMEM and 2 % FBS for additional 48 h. Cells were plated on glass coverslips in 35-mm-diameter Petri dishes containing the appropriate medium as described above. Coverslips were washed with phosphate-buffered saline (PBS), pH 7.2, and the cells fixed with 2 % paraformaldehyde containing 0.3 % Triton X-100 for 10 min at 37 °C, followed by washing three times with PBS. Cells were blocked with 2 % bovine serum albumin (BSA) and 5 % goat serum in PBS for 1 h at room temperature or overnight at 4 °C and then incubated for 4 h at room temperature in primary antibody diluted in blocking solution.

Circulating cells from stimulated Hirudo medicinalis

Ten leeches were stimulated, at the level of the 80^{th} superficial metamere, with injection of Matrigel (MG) (BD Biosciences, Mississauga, Canada) (300 μl) added with LPS. According to Grimaldi and coworker (2008) after 1 week MG implants were harvested from the animals, minced in small pieces using sterilized razor blades and mechanically dissociated with a micropipette in 400 µl of tissue culture. Cells were plated, cultured, maintained at 20 °C and examined histologically and immunocytochemically after 3 days from seeding. All cultures were performed in quadruplicate and processed as previously described.

Light microscopy, transmission electron microscopy (TEM) (standard procedure)

For routine TEM, collected circulating cells were fixed with 2 % glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 2 h. The pellet washed in 0.1 M Na-cacodylate buffer (pH 7.2), was post-fixed at 4 °C for 2 h with 1 % osmic acid in cacodylate buffer (pH 7.2). After standard dehydration in ethanol series, samples were embedded in an Epon-Araldite 812 mixture and

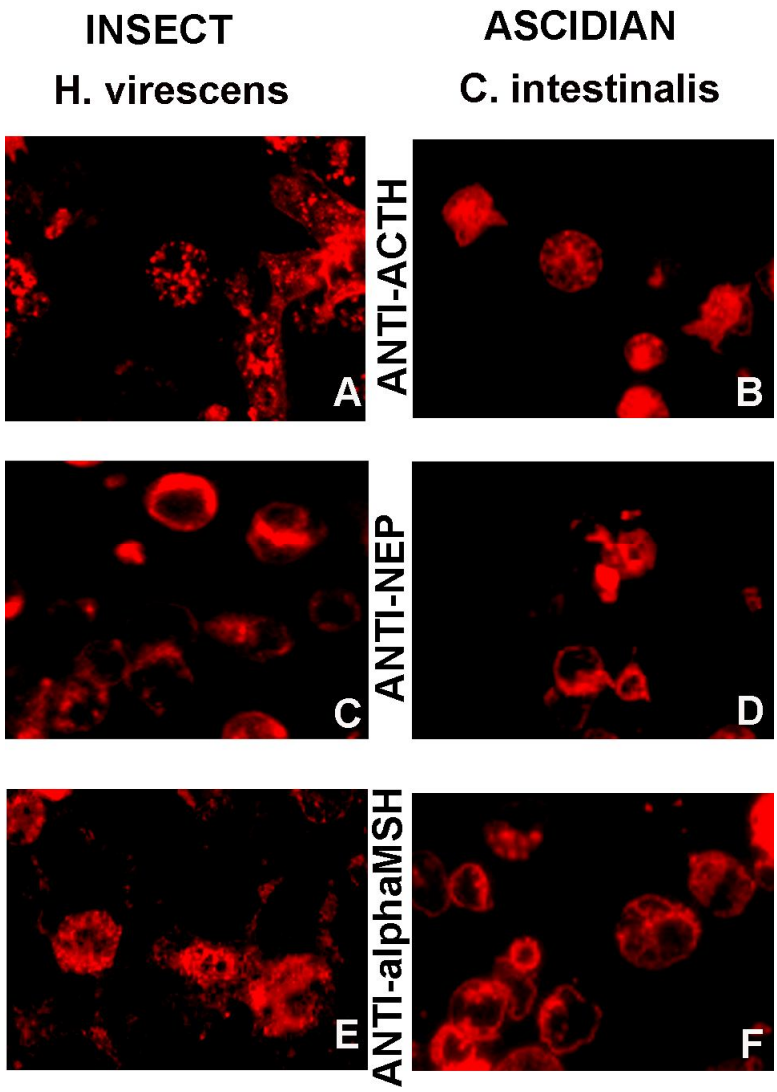

Fig. 2 Immunocytochemical characterization of insect/ascidian circulating hemocytes. (A-F) Fluorescence microscopy: expression of ACTH (A-B); NEP (C-D); α-MSH (E-F).

sectioned with a Reichert Ultracut S ultratome (Leica, Nussloch, Germany). Semithin sections were stained by conventional methods (crystal violet and basic fuchsin), and with May Grunwald Giemsa staining. Differential May Grunwald Giemsa staining depends on cytoplasmic pH (alkaline pH increases blue and acid pH pink or reddish tinge in the stained specimens), therefore is useful for a gross-identification of cells showing an increased reactive oxygen species production. Pictures visualized on a microscope Olympus BH2 (Olympus, Tokyo, Japan) were acquired with a DS-5M-L1 Nikon digital camera system. Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan).

Amyloid fibrils detection

Amyloid or amyloid-like structures can be recognized by different techniques. Amyloid fibrils exhibit strong affinity towards the dye Congo red and thioflavine T (Sipe and Cohen, 2000). Congo red and thioflavine T staining were performed according to Grimaldi *et al.* (2012). Specific fluorescence was visualized on a fluorescence microscope Olympus BH2 through a filter set (excitation wavelength of 465 nm emission). Images were acquired with a DS-5M-L1 Nikon digital camera system.

Melanin detection

Following the manufacturer's protocol (Bio-Optica, Milan, Italy), the Masson-Fontana silver stain was employed to demonstrate melanin deposition. Experiments were performed in triplicate.

Indirect immunofluorescence staining

Cryosections were treated for 30 min with PBS containing 2 % BSA before the primary antibody incubation (4 °C over night). The presence of ACTH,

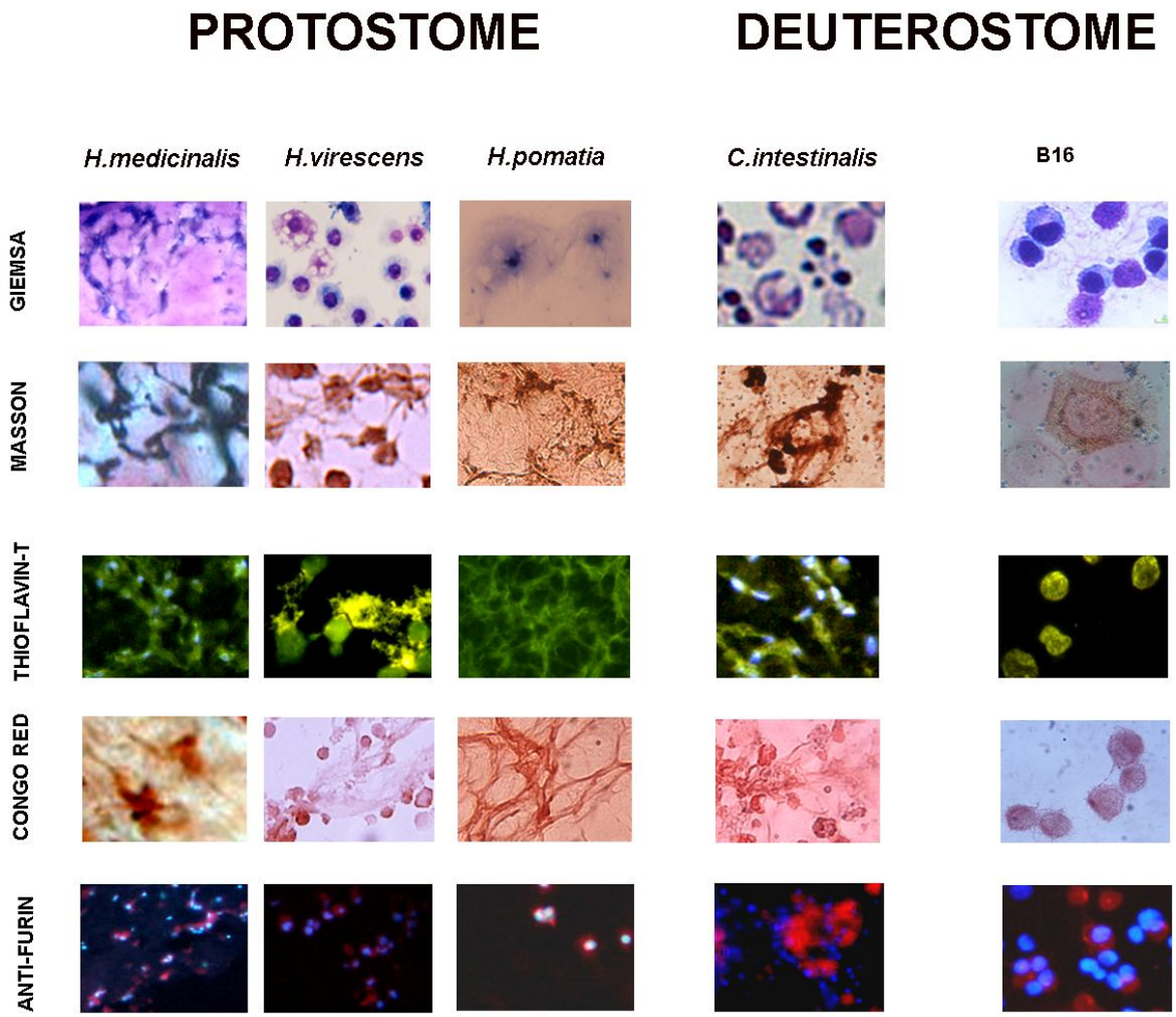

Fig. 3 Comparison between protostome and deuterostome. Cytoplasmic pH condition (Giemsa staining), melanin presence (Masson fontana technique), amyloid fibrils production (Thioflavine-T and Congo red staining), and furin expression are similar in *H. medicinalis* (Annelid), *H. pomatia* (Mollusc), *H. virescens* (Insect), *C.intestinalis* (Ascidian) circulating cells, and B16-F10 murine melanoma cell line (as positive control).

and its cleavage product α-MSH) (responsible for stimulation, production and release of melanin), due to NEP activity, and furin were assessed using the following primary antibodies: anti-human ACTH polyclonal antibody (1:50 dilution, SIGMA, Saint Louis, MO, USA); anti-human α-MSH polyclonal antibody (1:50 dilution, SIGMA); anti-CD10/CALLA (NEP) monoclonal antibody (clone 56C6, diluted 1:50, Thermo Scientific, Freemont, CA, USA), anti-furin polyclonal antibody (1:50 dilution, Santa Cruz Biothecnology, Santa Cruz, CA, USA). Incubations with suitable secondary antibodies conjugated with tetramethylrhodamine (TRITC) (1:200 dilution, Jackson, Immuno Research Laboratories, West Grove, Pennsylvania, USA) were performed for 1h in a dark moist chamber. Nuclei were eventually stained with 4',6-diamidino-2-phenylindole (DAPI, SIGMA, Italy). The PBS buffer used for washing steps and antibody dilutions contained 2 % bovine serum albumin (BSA). In control samples, primary antibodies were omitted, and samples were treated with BSA-containing PBS. Nuclei were stained by incubating for 15 min with 4-6-Diamidino-2-Phenylindole (DAPI, 0.1 g/ml in PBS). Coverslips were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA, USA); slides were observed on Olympus BH2 microscope (Olympus, Tokyo, Japan). Data were recorded with a DS-5M-L1 digital camera system (Nikon, Tokyo, Japan). Images were combined with Adobe Photoshop (Adobe Systems, Inc.).

Results

Starting from our previous data about the link between the melanin synthesis and the production

of amyloid fibrils, that template the pigment in activated insect *H. virescens* hemocytes (Falabella *et al.*, 2012; Grimaldi *et al.*, 2012), here we described and characterized the morpho-functional events linked to the production of amyloid fibrillar material in relation to melanin and the concomitant events that take place in the immune cells under stress condition in different invertebrates (Protostome and Deuterostome). For instance in insects and ascidians, melanin production (Figs 1 C, D) and amyloid fibril assemblage (Figs 1 E-H) [in the reticulum cisternae (Grimaldi *et al.*, 2012)] are always sustained by several conditions such as an overproduction of ROS responsible of pH variation (as previously validated by enzyme inhibition) (Figs 1 A, B), the presence of a furin-like proprotein convertase cleavage (Figs 1J, K) that it is well known liberates a fibrillogenic fragment in melanosomal biogenesis (Berson *et al.*, 2003). In addition we have observed that during this amyloid/pigment productive phase, a cross-talk between immune and endocrine systems occurs. These intercommunications are mediated by neuromodulators with the activation of stress-sensoring circuits to produce and release molecules such as ACTH and α-MSH (Figs 2 A-F). This scenario of cytoplasmic pH condition, amyloid fibrils production/melanin synthesis, and interaction between immune and neuroendocrine system (ACTH-α-MSH presence) as reported by Grimaldi and coworkers (2012) is validated also in different invertebrates (Fig. 3). On the whole, our data, here presented, and regarding *H. medicinalis* (Annelid), *H. pomatia* (Mollusc), *H. virescens* (Insect), *C. intestinalis* (Ascidian), and B16-F10 murine melanoma cell line (as positive control) were added to available data present in literature, and summarized respectively in the Figure 3 and Table 1.

Discussion

Extensive and deep studies were carried out on the multiple biochemical and morphological aspects involved in the protective responses of the immune system in invertebrates. Among the various potent weapons typical of a innate defense there are pro-PO system and granulocyte activations (Söderhäll and Smith, 1986; Jhoanson and Soderhall, 1989; Ashida and Yoshida, 1990; Roch *et al.*, 1992; Nappi and Vass, 1993; Fujimoto *et al.*, 1995; Yasuhara *et al.*, 1995; Pang *et al.*, 2004; Cammarata and Parrinello, 2009; Ballarin, 2012). The humoral pro-PO system that according to several authors corresponds in function to the activated complement (Soderhall, 1982; Johansson and Soderhall, 1989) was recorded in several *taxa* but it is not the most important defense mechanism for all invertebrates (Smith and Soderhall, 1991; Nappi and Ottaviani, 2000; Cerenius and Soderhall, 2004; Cerenius *et al.*, 2008; Cammarata and Parrinello, 2009; Ballarin, 2012). Independently from their phylogenetic position, invertebrates can produce melanin especially by humoral system or specifically by cellular population. In the first case massive production of melanin is due to the activity of pro-PO system that can be coupled with a cellular response lesser involved in pigment production. In the second one, the melanin synthesis is confined in cell where is concentrated in organules, the melanosomes. In any case it is interesting to underline that the production of melanin is always supported by the formation of amyloid fibrils, as well as the concurrent events, such as ACTH production, NEP increment and α-MSH formation.

In several invertebrates, such as artropods, melanin is massively produced in body cavity especially as pro-PO system product, while amyloid fibrils production is due to exocytosis of circulating cells (named in different ways as granulocytes or amebocytes) that are able to produce a huge amount of amyloid fibrils that adhere to the non-self driving the pigment accumulation close to the invaders, avoiding the toxic melanin dispersion in hemocelic environment (Ferrarese *et al.*, 2005; Falabella *et al.*, 2012; Grimaldi *et al.*, 2012). In other invertebrates and vertebrates there is a coupled productive system (melanin/amyloid fibrils) concentrated in a specific cell type, the melanocytes where melanin on amyloid fibrils is stocked in melanosomes (Fowler *et al.*, 2006). After stimulation, invertebrate cells engaged in melanin production, degranulate and their products flow close to the non-self or, as in vertebrates, the melanocytes convey towards superficial surface the pigment that are utilized as protection against UV.

Summarizing it is interesting to highlight that melanin employment is always coupled, from invertebrates up to man, with a physiological production of amyloid fibrils. The trade-off in utilizing the coupled system amyloid/melanin see a shift from the possibility to have two separated producers (humoral pro-PO system for melanin, and granulocytes for amyloid fibrils) as recorded in insects, echinoderms and ascidians with the following assemblage of the two products, up to a singular cellular producer of both products (pigment and amyloid fibrils) as in coelenterates, annelids, molluscs and vertebrates.

An additional striking aspect (in the previously mentioned *taxa*) refers to the cells involved in the production of amyloid fibrils that after cytoplasmic accumulation, are exocytozed to sustain melanin production. These cells, belonging to freely circulating hemocytes, show the same phenotype with a nucleus localized in central position, surrounded by large reticulum cisternae filled with fibrillar material, spatially organized in respect to a central electrondense core (Xing *et al.*, 2008; Grimaldi *et al.*, 2012).

All these features and related processes involved in amyloid fibrils/melanin synthesis in animal phylogenetically distant (*viz.*, cnidaria, molluscs, annelids, insects, ascidians and vertebrates) could be interpreted as evolutionary conserved. These shared innate immune responses could be interpreted in invertebrates as a basic event, constituting an integral component of immunity, independently deriving from a mix of cellular and humoral or from exclusive cellular responses, while in vertebrate could be interpreted as a modest and very restricted event of innate immunity. Indeed, in vertebrates the multiple and

Table 1

	Giemsa	Melanin	Amyloid		Furin	ACTH	α-MSH	References
			Thioflavine T	Congo red				
Cnidarians		***						(1, 2)
Annelids								
Polichets		***						(3, 4)
Oligochets		***						(5-8)
Hirudineans	***	***	***	***	***	***	***	(here) (9,10)
Molluscs	***	***	***	***		***	***	(here) (11-25)
Arthropods								
Crustaceans		***			***	***	***	(26-28)
Insects	***	***	***	***	***	***	***	(here) (29-33)
Echinoderms	***	***	***					(34)
Tunicates	***	***	***	***	***	***	***	(here) (35-38)
Cephalochordates		***						(39)
Vertebrates	***	***	***	***	***	***	***	(here) (40)

Available data present in literature has been summarized.
Cnidarians: (1, 2) Petes *et al.*, 2003 ; Mydlarz *et al.*, 2008. Annelids: (3) Porchet-Henneret and Verner, 1992; (4-10) Porchet-Henneret *et al.*, 1987; Beschin *et al.*, 1998; Fyffe *et al.*, 1999; de Eguileor *et al.*, 2000; Adamowicz, 2005; Prochazkova *et al.*, 2006; Grimaldi *et al.*, 2008. Molluscs: (11-25) Ottaviani and Cossarizza, 1990; Ottaviani, 1983, 2006; Ottaviani *et al.*, 1990, 1993; Gourdon *et al.*, 1993; Giamberini *et al.*, 1996; Ottaviani and Franchini, 1998; Matricon-Gondra, 1999; Gorbushin *et al.*, 2007; Koropatnick *et al.*, 2007; Martin *et al.*, 2007; Mahilini *et al.*, 2008; Novoa *et al.*, 2011. Arthropods: (26-32) Soderhall and Smith, 1986; Johansson and Soderhall, 1989; Nappi and Vass, 1993; Hoffman and Reichart, 2002; Ferrarese *et al.*, 2005; Gallo *et al.*, 2011; Falabella *et al.*, 2012; Grimaldi *et al.*, 2012. Ehinoderms: (34) Canicattì and Seymur, 1991. Tunicates: (35) Ballarin, 2008; (36) Ballarin, 2012; (37) Cammarata and Parrinello, 2009; (38) Hirose, 2003. Cephalochordates: (39) Pang *et al.*, 2004. Vertebrates: (40) Fowler *et al.,* 2006

multifaceted responses belonging to acquired immunity can mask the basic innate responses due to the presence of numerous modulate answers against the non-self leading to a precise discrimination of individual pathogenic species. Another aspect that must be considered is the evidence of bidirectional messages between immune and neuroendocrine system. Thus amyloid/melanin production is close associated to ACTH/α-MSH production, emerging here as molecule overespressions. Their presence and function related to stress responses leading to pigment production reflect and confirm their ancient phylogeny (Wilder, 1995; Ottaviani and Franceschi, 1996).

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