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Cytomorphology and PCNA expression pattern in bivalves *Mytilus galloprovincialis* and *Cerastoderma edule* with haemic neoplasia

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ABSTRACT: Haemic neoplasia (HN) is a pathologic condition reported in several bivalve species in different geographic areas. In this study we describe the cytomorphological features and the proliferative behaviour, assessed by the proliferating cell nuclear antigen (PCNA), of HN in common cockle *Cerastoderma edule* and Mediterranean mussel *Mytilus galloprovicialis*. In mussels the presence of at least 5 types of atypical haemocytes was detected, including A- and B-type cells, previously described in *M. edulis* and *Mytilus* sp., with predominance of A-type cells in early phases of the disease and B-type cells in more advanced stages. PCNA immunostaining was positive for 97 to 100% of the neoplastic cells, with both cytoplasmic (A cells) and nuclear patterns (B cells). Conversely, in *C. edule* there was no distinctive morphological cell sub-population, and staining atypical haemocytes with PCNA (range 93 to 100%) showed nuclear expression in early phases of disease and cytoplasmic expression in more advanced stages. The above findings suggest distinct histo-pathogenetic pathways for HN in mussels and common cockles.

KEY WORDS: Mediterranean mussel · Common cockle · Haemic neoplasia · Proliferating nuclear antigen · Proliferation indices

INTRODUCTION

Two predominant types of neoplasia have been reported in marine molluscs, viz. disseminated neoplasia, also called leukemia or haemic neoplasia (HN), and gonadal neoplasia, (Peters et al. 1994, Barber 2004, Carella et al. 2009). In HN, neoplastic cells are represented by 'atypical' haemocytes, a term which incorporates the sum of the differences in morphological, biochemical and functional features relative to normal cells (De Vico & Carella 2012a). Atypical haemocytes display high nucleus to cytoplasm ratios, diffuse chromatin patterns and pleomorphic nuclei, and usually infiltrate tissues and organs of affected individuals (Auffret & Poder 1986, Villalba et al. 2001).

HN has been described for several bivalve species from different geographical origins, reported in association with mass mortality events (Elston et al. 1992, Peters et al. 1994, Alonso et al. 2001, Villalba et al. 2001). In the Mediterranean mussel *Mytilus galloprovincialis*, very few cases have been reported, with limited cytological and functional features described (Tiscar et al. 1990, Zizzo et al. 1991, Figueras et al. 1991a,b, Ciocan & Sunila 2005). In common cockles *Cerastoderma edule*, HN was first observed in 1982 in Cork Harbour, Ireland (Twomey & Mulcahy 1988), and was also found in cockle populations from the

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northern coast of Brittany, France (Auffret & Poder 1986, Poder & Auffret 1986), with high prevalence in animals from Galician waters (NW Spain; Ordás & Figueras 2005, Le Grand et al. 2010).

From a comparative point of view, neoplastic bivalve haemocytes provide excellent *in vivo* and *in vitro* models for some human lymphomas and other unrelated human and animal cancers (Walker et al. 2011). In this context, molluscan HN is considered a phenotypically similar proliferative disease in the different shellfish species involved (e.g. *Crassostrea virginica*, *Mytilus* spp. and *Ostrea edulis*; Walker et al. 2011). However, differences in neoplastic cell morphology, along with descriptions of neoplastic haemocyte subtypes, have frequently contradicted this assumption (Lowe & Moore 1978, Green & Alderman 1983).

Here we describe for the first time the cytomorphological features and the proliferating cell nuclear antigen (PCNA) expression pattern (assessed by immunohistochemical detection of PCNA, the auxiliary protein of DNA polymerase-δ and ε) of HN in *Mytilus galloprovincialis* from the Mediterranean Sea (Gulf of Naples, southern Italy). We also compared the above findings in *M. galloprovincialis* to those of HN cells in *Cerastoderma edule* from Galician waters (Carril, Vilagarcía de Arousa, Spain).

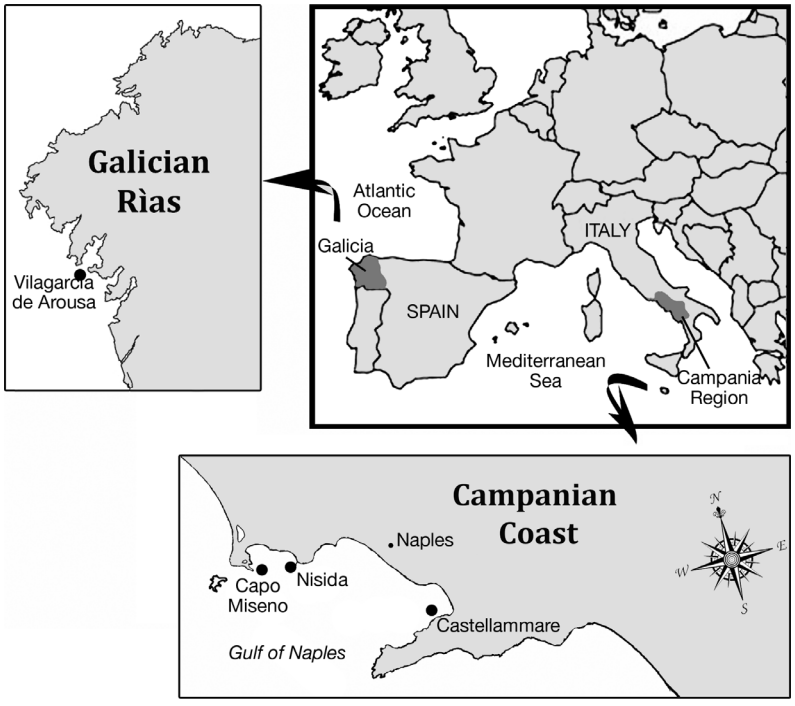

Fig. 1. Campanian coast (Italy) and Galician Rìas (Spain) with sampling stations

MATERIALS AND METHODS

A total of 20 bivalves affected by HN (10 *Mytilus galloprovincialis* and 10 *Cerastoderma edule*) were employed in this study. The affected *M. galloprovincialis* were collected in the Gulf of Naples (southern Italy, Campania region) in summer 2010 at 3 mussel farms. Cases of HN were recorded at Nisida (3 individuals), Capo Miseno (4 individuals) and Castellammare (3 individuals), retrieved from the archival material of the Department of Biological Sciences, University of Naples Federico II. Affected *C. edule* were sampled in Galician waters in 1999, at Carril, Vilagarcía de Arousa, Spain, retrieved from archival material of the Instituto Investigaciòn Marina, Spain (Fig. 1).

Tissues were fixed in Davidson's solution for at least 48 h, immediately embedded in paraffin blocks, sectioned at 5 µm with a rotary microtome, stained with haematoxylin and eosin (H&E) and observed under light microscopy.

In affected *Mytilus galloprovincialis*, HN staging was evaluated according to Lowe & Moore (1978) and Galimany & Sunila (2008), with slight modifications: Stage 1 – Focal (digestive gland location); Stage 2 – Intermediate (neoplastic cells infiltrating other organs); Stage 3 – Terminal (loss of tissue architecture). Staging for *Cerastoderma edule* HN was evaluated according to Le Grand et al. (2010).

For each case, individual tumour cells were selected by morphological criteria according to specific literature for molluscs, when available (Lowe & Moore 1978); any cell type not described previously was carefully recorded, counting at least 200 cells for each case, and tentatively classified according to their main morphological features, following, when appropriate, the nomenclature used for describing morphologically similar cells detected in some lymphoproliferative disorders of higher vertebrates (Lanza 1980, Vernau et al. 1992, Ponce et al. 2010). The mitotic index (MI) was determined by counting, for each sample, the number of mitotic figures per 1000 neoplastic cells (De Vico et al. 1996).

PCNA immunohistochemistry and quantification were made according to previous reports (Maiolino et al. 1995, De Vico et al. 1996) with slight modifica-

tion, using a mouse monoclonal antibody against PCNA, clone PC10 (Sigma), which was already successfully tested to localize proliferating cells in several mollusc species, including *Mytilus galloprovincialis* (Marigómez et al. 1999, Hanselmann et al. 2000, Harris et al. 2006, Franco et al. 2010). Briefly, after deparaffinization of the tissue sections, endogenous peroxidase was blocked by incubation in H_2O_2-methanol (4:1) for 20 min. After hydration, the sections were incubated with sodium citrate buffer (pH 6.0) in a microwave for 5 min at 700 W, in order to unmask the antigens and epitopes. Subsequently, the sections were allowed to cool down to room temperature, and after several washes in phosphate-buffered saline (PBS), sections were treated with 10% goat serum, permeabilized with PBS/BSA 1% and incubated overnight at 4°C with the primary antibody (dilution 1:300). Sections were rinsed in PBS and treated with the corresponding biotinylated secondary antibody for 20 to 30 min at room temperature. Sections were then rinsed in PBS, and the detection was performed via DAB (diaminobenzidine; LSAB-Dako). The sections were counterstained with Carazzi haematoxylin for 5 min at room temperature, dehydrated through graded alcohols and mounted for microscopical examination.

RESULTS

In both *Mytilus galloprovincialis* and *Cerastoderma edule*, the neoplastic condition was characterized by infiltration and replacement of the vesicular connective tissues by enlarged, mitotically-active, atypical haemocytes (Fig. 2).

Cell morphology was different in the 2 species. In mussels, the presence of at least 5 morphological types of atypical haemocytes was recorded: 2 of them corresponded to the previously described A and B types (Lowe & Moore 1978, Green & Aldermann 1983), and the others (indented/cleaved cells, binucleated cells and multinucleated giant cells; Lanza 1980, Vernau et al. 1992, Ponce et al. 2010), described here for the first time in *Mytilus galloprovincialis*, appeared as A or B cell subtypes. Moreover, a normal inflammatory component, greatly variable in percentage and comprising hyaline or granular haemocytes, was also present (see Fig. 2A–G). A-type cells were ovoid in shape, in some cases exhibiting marked pleomorphism (polymetrism and polymorphism), vesicular nuclei and evident nucleoli. B-type cells were rounded, with nuclei showing a dense chromatin pattern (Fig. 2A,B).

According to the staging, 3 cases were classified as early lesions, with neoplastic cells underlying the stomach epithelium, mainly composed of A cells (78–88%). Four were at the intermediate level (Stage 2) of neoplasia, characterized by small isolated scattered foci of both A and B cells, distributed in different percentages, in gills, visceral mass, mantle and kidney; at this stage, necrosis of digestive tubules was recorded in 2 cases. Advanced disease (Stage 3) essentially consisted of massive proliferation of the abnormal cells, mainly B type (87 to 90%), replacing all of the vascular spaces with relatively few normal haemocytes, and extensive loss of tissue architecture. Moreover, the MI varied from 4 to 10%, accompanied in some cases by atypical mitosis. The other observed types of cell showed variable percentages over the course of the disease: indented cells ranged from 1 to 8%, binucleated cells from 1 to 5% and multinucleated cells from 1 to 4%, respectively (Fig. 3).

In common cockles, cellular morphology was different, with no distinctive cell sub-population of A and B types: neoplastic haemocytes were large cells, with round to oval vesicular nuclei and one or more evident nucleoli. However, multinucleated giant cells, indented cells and binucleated cells were also recognized (Figs. 2H–L & 3). The MI ranged from 4 to 11%.

PCNA-positive cells ranged from 93 to 100% in all examined cases. In mussels, PCNA in A cells was mostly confined to the cytoplasmic level, whereas B cells had mostly nuclear positivity. In contrast, neoplastic cells in common cockles showed predominantly a nuclear PCNA positivity in early phases of the neoplastic condition, with cytoplasmic positivity in more advanced stages (Fig. 4). In both mussels and cockles, binucleated cells sometimes showed nuclear PCNA labelling, while other cells, like multinucleated giant cells and indented/cleaved cells, were PCNA negative (Fig. 4B).

DISCUSSION AND CONCLUSIONS

Two major hypotheses have been formulated regarding the histogenesis of A- and B-type cells in HN from the bivalve species in which they have been recognized: Moore et al. (1991) suggested that in HN of *Mytilus edulis*, the A and B cell types could represent 2 distinct cell lineages; in contrast, Lowe & Moore (1978) suggested that A and B cells could represent consecutive developmental stages of a single cell lineage during the progression of the disease. Our data in *M. galloprovincialis* based on disease staging com-

bined with the different PCNA staining patterns of the cells seem to support the latter hypothesis. In fact, early stages of the disease in our study were characterized by the large predominance of A-type cells only, while in Stage 2, B cells increased and progressively replaced A cells in Stage 3. A-type cells in our case showed prevalent cytoplasmic PCNA labelling, whereas B cells showed primarily nuclear PCNA immunostaining. On the other hand, in common cockles, neoplastic haemocytes had some morphological features resembling the A cell type of mussels (i.e. vesicular round to oval large nuclei with evident nucleoli), but showed nuclear PCNA immunostaining in early phases of the disease, while in advanced stages they showed predominantly cytoplasmic labelling. It is known that PCNA is also expressed during the cell cycle in proliferating cells of molluscs (Marigómez et al. 1999, Hanselmann et al. 2000, Harris et al. 2006, Franco et al. 2010): PCNA levels increase during the G1-S phase of the cell cycle, and usually localize in the nucleus, being involved in cell proliferation control and DNA repair (Paunesku et al. 2001). However, recent studies in human granulocytes suggest that PCNA could be also involved in suppressing apoptosis when localized in the cytoplasm, where it binds to and inhibits the procaspases, which are key molecules in apoptosis control (Witko-Sarsat et al. 2010); interestingly, this pattern has also been ob-

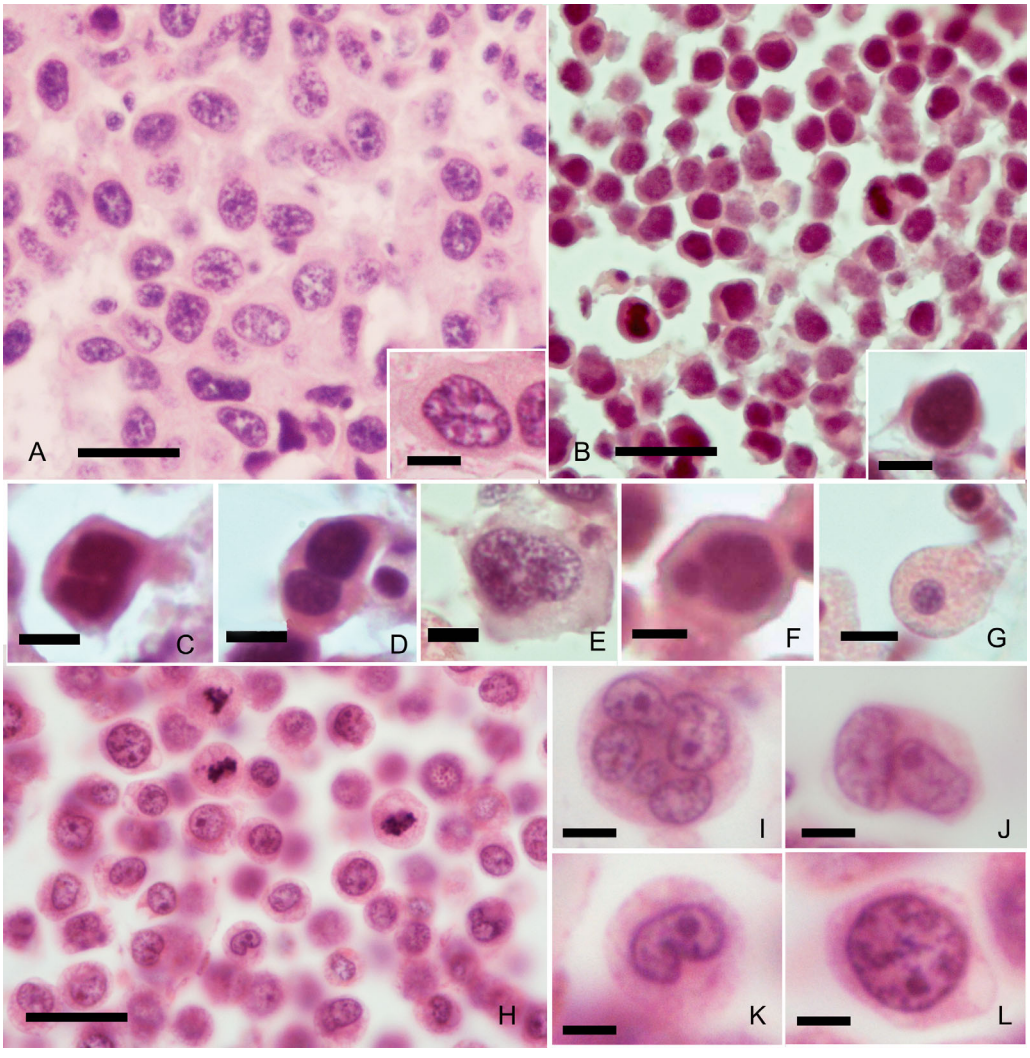

Fig. 2. Neoplastic cell morphology in (A–F) *Mytilus galloprovincialis* and (H–L) *Cerastoderma edule*. (A,B) A- and B-type cells as described by Lowe & Moore (1978). (C) Cell with cleaved nuclei. (D) Binucleated cell. (E) Multinucleated giant cell showing at least 3 nuclei, disperse chromatin and visible nucleoli. (F) Cell with high nuclear/cytoplasm ratio, also showing a nuclear bleb. (G) Normal haemocytes: ialonocyte (smaller) and granulocyte. (H–L) Typical anisocariosis and aniso-cytosis of neoplastic cells also accompanied by few giant (I), binucleated (J), indented (K) and mononucleated cells (L). Scale bars = (A,B,H) 25 µm, (C–G, I–L) 5 µm

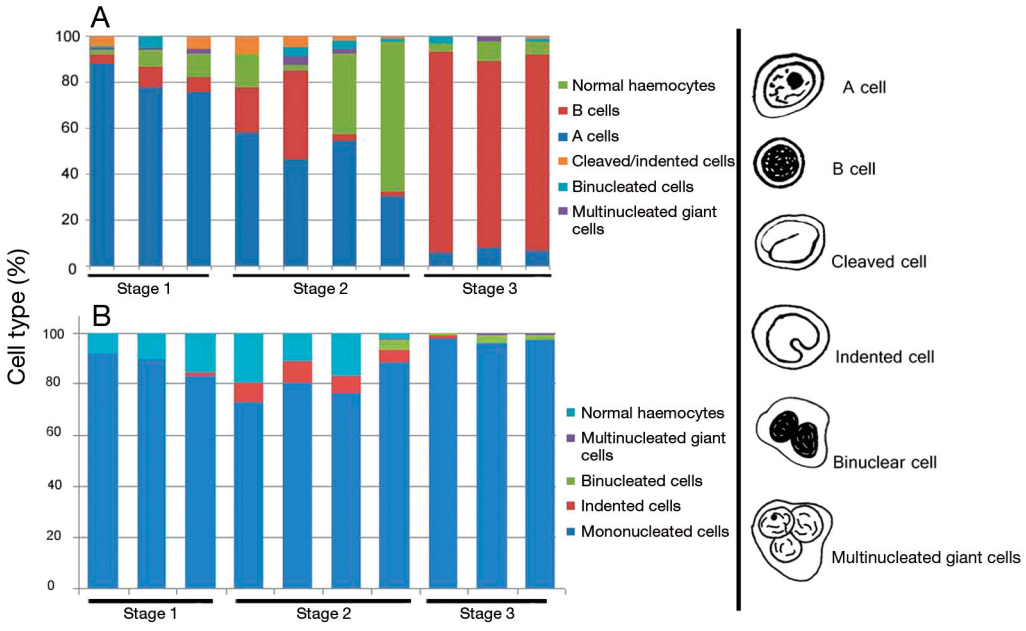

Fig. 3. *Mytilus galloprovincialis* and *Cerastoderma edule.* Cell type description and percentage over the course of haemic neoplasia (Stages 1–3) in (A) mussels and (B) common cockles. Modified from Lanza (1980) and Bartl et al. (1984)

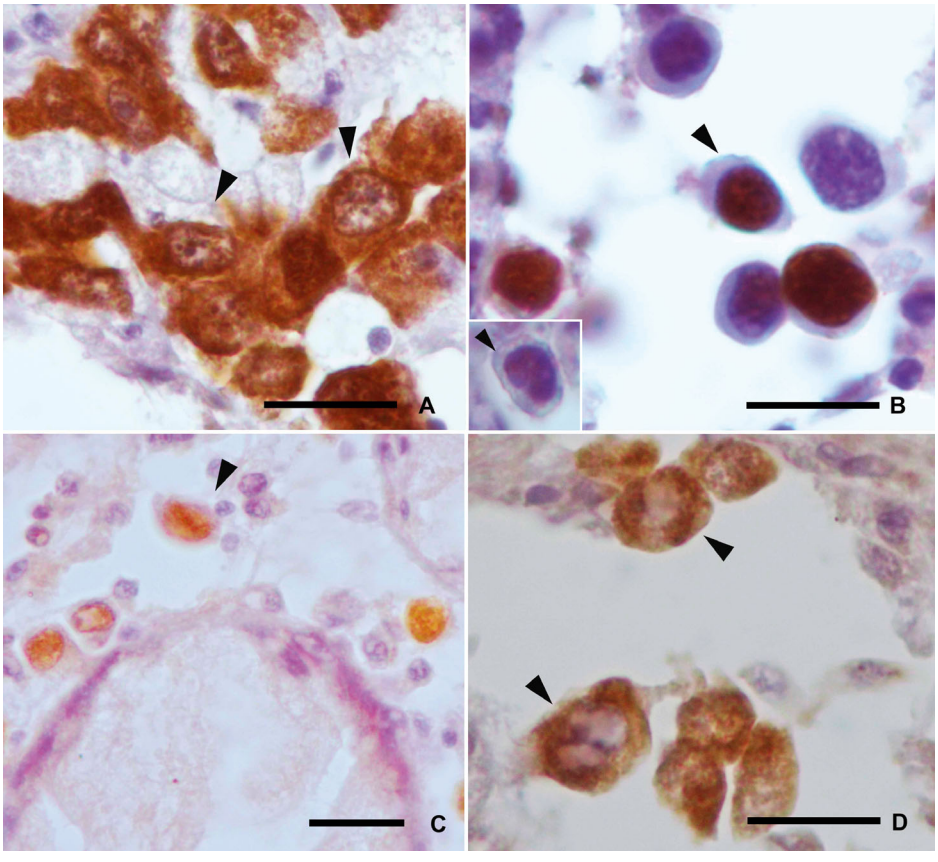

Fig. 4. *Mytilus galloprovincialis* and *Cerastoderma edule.* Proliferating cell nuclear antigen (PCNA) assay of neoplastic cells of mussels and cockles. (A,B) PCNA shows positivity at the cytoplasmic level in A-type cells (A) and at the nuclear level in B-type cells (B), with no positivity evident in indented cells. (C,D) Nuclear positivity in early phase of disease (C) and cytoplasmic in more advanced stages (D). Scale bars = 25 µm

served in human Hodgkin's lymphoma (Benjamin & Gown 1991). In this regard, blocking apoptosis and activating proliferation are well known mechanisms involved in early carcinogenesis and/or in neoplastic progression (De Vico & Carella 2012a). Thus, in our case, one can speculate that distinct pathogenetic pathways could be responsible for HN in mussels and common cockles. In fact, the presence of cytoplasmic localization of PCNA in A cells in mussels suggests that this phenomenon could play a role in early phases of the disease, allowing survival of initiated haemocytes by escaping apoptosis, while B cells could represent a sub-population which acquired a proliferative advantage over the others, under unknown promoting/progressing factors. Conversely, in common cockle, neoplastic haemocytes displaying a nuclear PCNA positivity could represent a highly proliferating population of initiated cells in early phases of HN, while cytoplasmic PCNA positivity could promote tumour progression in advanced stages by blocking apoptosis. The latter hypothesis seems to be strongly supported by a recent paper by Díaz et al. (2013) on the promoting/progression role of escaping apoptosis by neoplastic haemocytes in advanced phases of HN in *Cerastoderma edule*. As far as indented/cleaved cells and multinucleated giant cells detected in our study are concerned, they have been classified according to the nomenclature used for morphologically similar cells detected in some peculiar lymphoproliferative disorders of higher vertebrates where cellular subtypes have well recognized diagnostic relevance (Lanza 1980, Valli et al. 1981, Vernau et al. 1992, Ponce et al. 2010). For mussel specimens, in our case, these cells could be considered as subtypes of the observed A and B types.

Given the differences in the physiology and histogenesis of immune cell lineages between vertebrates and invertebrates (De Vico & Carella 2012b), we cannot attempt any assumptions about their comparative biological significance. However, according to their PCNA expression patterns, it could be speculated that these cells represent non-proliferating cell subpopulations, whose origin and role should be elucidated in further studies.

The above findings strongly support the hypotheses of distinctive morphological and histo-pathogenetic behaviours of HN in each of the 2 examined species even considering the small number of samples examined. Further molecular studies should be conducted in order to better elucidate the biochemical and genetic backgrounds underlying the differential pathogenesis of these interesting animal models of neoplasia.

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