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REGULAR ARTICLE

Concholepas hemocyanin biosynthesis takes place in the hepatopancreas, with hemocytes being involved in its metabolism

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Abstract Hemocyanins are copper-containing glycoproteins in some molluscs and arthropods, and their best-known function is O_2 transport. We studied the site of their biosynthesis in the gastropod *Concholepas concholepas* by using immunological and molecular genetic approaches. We performed immunohistochemical staining of various organs, including the mantle, branchia, and hepatopancreas, and detected *C. concholepas* hemocyanin (CCH) molecules in circulating and tissue-associated hemocytes by electron microscopy. To characterize the hemocytes, we purified them from hemolymph. We identified three types of granular cells. The most abundant type was a phagocyte-like cell with small cytoplasmic granules. The second type contained large electron-dense granules. The third type had vacuoles containing hemocyanin molecules suggesting that synthesis or catabolism occurred inside these cells. Our failure to detect *cch*-mRNA in hemocytes by reverse transcription with the polymerase chain reaction (RT-PCR) led us to propose that hemocytes instead played a role in CCH metabolism. This hypothesis was supported by colloidal gold staining showing hemocyanin molecules in electron-dense granules inside hemocytes. RT-PCR analysis, complemented by in situ hybridization analyses with single-stranded antisense RNAs as specific probes, demonstrated the presence of *cch*-mRNA in the hepatopancreas; this was consistent with the specific hybridization signal and confirmed the hepatopancreas as the site of CCH synthesis. Finally, we investigated the possibility that CCH catabolism in hemocytes was involved in the host immune response and in the generation of secondary metabolites such as antimicrobial peptides and phenoloxidase.

Keywords Hemocyanin · Biosynthesis · Hepatopancreas · Hemocytes · *Concholepas concholepas* (Mollusca, Gastropoda: Muricidae)

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Introduction

Hemocyanins are found in molluscs and arthropods as huge multi-subunit circulating glycoproteins that transport O_2 molecules via their binuclear active site. The active site contains two copper atoms bound directly to the polypeptide chain via highly conserved histidine residues (van

Holde and Miller 1995; van Holde et al. 2001). These proteins are part of the type-3 group of copper proteins that includes phenoloxidases and tyrosinases. However, their physiological functions differ; whereas hemocyanins function as O_2 carriers, the other proteins are involved in wound healing and in primary immune defence in all organisms in which they have been assessed (Decker and Tuczek 2000).

Mollusc hemocyanins exhibit a complex system of self-assembly and a sophisticated quaternary structure formed by ten subunits that are organized as a hollow cylindrical array as a decamer, with a molecular mass of approximately 4 MDa. In some molluscs, such as gastropods, the decamers are associated in pairs called didecamers, which constitute colossal molecules with molecular masses of approximately 8 MDa. The polypeptide chains or subunits of these proteins are large (approximately 400 KDa each) and folded into a series of eight globular domains called functional units (FUs; 50 KDa) connected by linker peptide strands. Each FU has two well-separated copper atoms: one called the A-site, which is located towards the N-terminus, and one called the B-site, located downstream of the polypeptide (van Holde et al. 2001).

Hemocyanins have attracted much interest in biomedicine and biotechnology, mainly because of their powerful immunogenic capacity in mammals. The most commonly used hemocyanin is obtained from *Megathura crenulata* and is known as keyhole limpet hemocyanin (KLH; Harris and Markl 1999). As a result of its immunogenic capacity, KLH has been extensively used as a carrier protein to produce specific antibodies against hapten molecules and peptides (Chiarella et al. 2010). This protein has also been employed as a carrier adjuvant in the development of experimental vaccines against pathogens and cancer (Musselli et al. 2001). Moreover, KLH itself has been used as a therapeutic agent in the treatment of superficial bladder cancer (Lamm et al. 2000; Markl et al. 2001; Moltedo et al. 2006).

In our laboratory, we have focused our attention on the hemocyanin isolated from the edible gastropod *Concholepas concholepas*, which is distributed on the west coast of South America between Callao in Perú and Cape Horn in Chile (Huaquín and Garrido 2000). Several hemocyanins from various species of molluscs have been characterized. The *C. concholepas* hemocyanin (CCH), however, has an unusual organization. In gastropods that carry two types of hemocyanin subunits, the decamers are formed by only one type; thus, they comprise homodecamers that associate in pairs, named homodidecamers, as observed in *Megathura crenulata* (Swerdlow et al. 1996), *Haliotis tuberculata* (Meissner et al. 2000), and *Rapana thomasiana* (Gebauer et al. 1999). By contrast, in *C. concholepas*, which contains two subunits, the decamers are formed by two types of intermingled subunits that form a heterodecamer. Consequently, their association in pairs results in heterodidecamers (De Ioannes et al. 2004; Becker et al. 2009). Thus, the structural differences between these hemocyanins raise problems that remain to be solved with regard to their synthesis. For example, we currently do not know whether the cells that synthesize each type of subunit are different, or whether the two types of subunits are synthesized together in the same cells. To answer these questions in *C. concholepas*, we must first determine the location of hemocyanin synthesis.

KLH, CCH, and several other gastropod hemocyanins are under extensive study in order to determine their biochemical and immune properties (Moltedo et al. 2006; Siddiqui et al. 2007; Dolashka-Angelova et al. 2007; Tchorbanov et al. 2008; Idakieva et al. 2008; Becker et al. 2009; Jaenicke et al. 2010), to resolve their structure (Hartmann et al. 2004; Gatsogiannis and Markl 2009), and to identify their genes (Miller et al. 1998; Lieb et al. 2001, 2004; Altenhein et al. 2002; Bergmann et al. 2006, 2007). The biosynthesis of hemocyanins in molluscs has been studied previously (Ruth et al. 1988, 2000; van Holde and Miller 1995; Taylor and Anstiss 1999; Albrecht et al. 2001; Streit et al. 2005). Several experimental studies involving immunohistochemical and cytochemical techniques with anti-hemocyanin antibodies together with in situ hybridization assays using RNA probes have demonstrated that hemocyanin biosynthesis in molluscs occurs in various organs depending on the species studied. Thus, in cephalopods such as *Octopus dofleini* and *Octopus vulgaris*, the synthesis of hemocyanin is limited to the branchial gland (Miller et al. 1998). In contrast, this function resides in the hepatopancreas of the tetrabranchiate *Nautilus pompilius* (Ruth et al. 1996). On the other hand, roghocytes, which are also known as pore cells, have been shown to synthesize hemocyanin in several pulmonate gastropods molluscs, such as *Lymnaea stagnali* (Sminia and Boer 1973), *Helix aspersa* (Sminia and Vlugh-van Dallen 1977), and *Haliotis tuberculata* (Albrecht et al. 2001). In addition, roghocytes are involved in hemocyanin homeostasis in *Sepia officinalis* (Beuerlein et al. 2002a). These cells are also the major sites of copper storage in its granular form (Dallinger et al. 2005).

The aim of the present study has been to identify the location of hemocyanin biosynthesis in adult animals of *C. concholepas* via immunohistochemical and cytochemical techniques with polyclonal anti-CCH antibodies complemented by in situ hybridization analyses with single-stranded cRNA probes and by reverse transcription with the polymerase chain reaction (RT-PCR) analysis. The *cch* primer design was based on alignments among all of the reported conserved sequences that code for the Cu-B site of mollusc hemocyanins (Lang and van Holde 1991; Miller et

al. 1998; Lieb et al. 2001, 2004; Keller et al. 1999; van Holde et al. 2001; Altenhein et al. 2002; Stoeva et al. 2002). The results indicate that the hepatopancreas is the location of hemocyanin biosynthesis in *C. concholepas*. In addition, the findings of this study strongly suggest that hemocytes play a number of significant roles in CCH metabolism.

Materials and methods

Animals

Live adult individuals of *Concholepas concholepas* (commonly known as Loco) were harvested from the Pacific Ocean (Quintay Bay; 33° 8′ S 71° 48′ W; 5th Region of Chile). The specimens were immersed in seawater and transported live to the laboratory. Tissues were dissected and processed as described below.

Peroxidase/anti-peroxidase method for light microscopy

Tissues were fixed with freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 24 h at room temperature. The samples were then dehydrated in a graded alcohol series and embedded in paraffin (Paraplast X-TRA Tissue Embedding Medium; Oxford Labware, USA). Tissue sections (5 μm) were cut, placed on gelatin-coated slides, and stored at room temperature. After paraffin removal with xylene (2×10 min), tissue sections were rehydrated in a graded alcohol series with two changes (10 min) at each concentration (100%, 96%, 90%, 87%, 70%, 50%). The sections were then washed with PBS and treated with absolute methanol and 3% (v/v) hydrogen peroxide (Merck, Germany) for 30 min. After being washed with PBS (2×5 min), the tissue sections were incubated for 1 h at room temperature with blocking buffer consisting of 1% bovine serum albumin (BSA), 5% low-fat milk, and 0.3% Triton X-100 in PBS. Immunostaining assays were performed by using the peroxidase/anti-peroxidase (PAP) method (Sternberger et al. 1970) with some modifications (Figueroa et al. 2005). The sections were incubated overnight with rabbit anti-CCH antibodies at dilutions of 1:500 to 1:2,500. Subsequently, they were incubated with anti-rabbit IgG from goat (Rockland, USA) and PAP complexes (Rockland). Peroxidase activity was detected with 0.1% (w/v) 3,3′-diaminobenzidine (BRL; Rockville, USA) and 0.03% (v/v) hydrogen peroxide (Merck, Germany). To determine the specificity of the immunoreactions, control assays were conducted without the addition of specific antibody. Some sections were stained with hematoxylin and eosin (HE) to observe and ensure adequate tissue preservation.

Transmission electron microscopy

Small portions extracted from the mantle, branchia, and hepatopancreas were excised and fixed in freshly prepared 2% glutaraldehyde (Polysciences, USA) in 0.1 M cacodylate buffer (pH 7.4) for 24 h. Hemocytes were collected from hemolymph by bleeding the animals at 4°C via several diagonal cuts generated on the mantle and foot of the mollusc under germ-free conditions and subsequently filtered through a glass mesh (De Ioannes et al. 2004). Hemocytes were collected by centrifugation at 1,500*g* for 10 min at 4°C and fixed as described above. Each sample was post-fixed with buffered osmium tetroxide solution for 1 h, dehydrated in graded acetone without rinsing, and embedded in Epon (Polysciences; Luft 1961). Thin sections (400-500 Å) were then generated and double-stained with 4% uranyl acetate in methanol and lead citrate (Reynolds 1963). The negatively stained hemocyanin sample was treated according to a previously described method (De Ioannes et al. 2004). The preparations were analyzed and photographed at 80 kV with a Philips TECNAI 12 electron microscope (Electronic Microscopy Facility, Pontificia Universidad Católica of Chile).

Immunogold labeling for electron microscopy

Tissue samples were fixed for 24 h in fresh 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The tissue samples were then dehydrated in ethanol, embedded with LR White resin, and cured at 65°C for 18 h. Thin sections of the samples were placed on carbon-coated Formvar nickel grids and allowed to dry overnight prior to staining. The grids were floated face down onto sequential drops of labeling and washing solutions. Typically, we used 100-μl drops per grid for every solution except the antibody solution, for which only 5 μl per grid was sufficient. Briefly, the tissue samples were blocked with 1% BSA in PBS for 1 h at room temperature to avoid nonspecific binding. The samples were then incubated for 1-2 h with anti-*Concholepas* hemocyanin rabbit serum developed in our lab at dilutions ranging from 1:15,000 to 1:45,000 in PBS containing 1% BSA. The samples were washed with 0.1% BSA and 0.01% Tween-20 in PBS and incubated for 1 h at room temperature with goat anti-rabbit IgG-gold (10-nm diameter particles; Sigma, USA). Subsequently, they were washed and stained with uranyl acetate in methanol and lead citrate as described above. For controls, the antibody against CCH was omitted from the assay.

Isolation of total RNA from various *C. concholepas* tissues

Samples were obtained from individually extracted pieces of the hepatopancreas, mantle, branchia, and hemocytes and

frozen in liquid nitrogen to preserve the RNA quality. Total RNA was isolated and purified from the various tissues by using an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

RT-PCR analysis

Purified total RNA (1-5 μg) was incubated at 70°C for 10 min for denaturation. Each RT-PCR was initiated by adding 200 U reverse transcriptase (Moloney Murine Leukemia Virus; Invitrogen, USA) followed by incubation at 42°C for 1 h. To inactivate the enzyme, the samples were further incubated at 70°C for 15 min. The cDNAs were diluted five-fold. The PCR was performed by using 2-4 μl of each sample under standard reagent conditions and 5 U/μl *Taq* DNA polymerase (Invitrogen). The following programme was used: 94°C for 3 min, followed by 30 cycles at 94°C for 1 min and 54°C for 1 min, and a final extension at 72°C for 5 min.

Construction of hemocyanin cRNA probes

Previously, we had cloned a 1.17-kb cDNA corresponding to the Cu-B center from *Concholepas concholepas* hemocyanin, which was used as a template for the synthesis of cRNA by using in vitro transcription (run-off assay). The cRNA was labeled with digoxigenin (Roche Diagnostic, Germany). A cloned β-actin gene from *C. concholepas* served as an internal control. The plasmids were digested by incubation at 37°C for 3 h with *Bam*HI to generate a sense probe or *Eco*RV (both restriction enzymes from Invitrogen) for an antisense probe. The digested DNA was then cleaned by using a MiniElute kit (Qiagen). The run-off was conducted with T7 RNA polymerase (sense probes) or Sp6 RNA polymerase (antisense probes) at 37°C for 2 h. The probes were analyzed by electrophoresis in 1.2% agarose gels containing 6.6% formaldehyde in 50 mM MOPS (pH 7) and observed under UV light after being stained with ethidium bromide (1 μg/ml).

In situ hybridization

For in situ hybridization, a previously described procedure was used (Figueroa et al. 2005). Deparaffinized sections on slides were immersed for 10 min in cold 2× SSC (3 M sodium chloride; 0.3 M sodium citrate, pH 7.0) and incubated for 1 h at room temperature in a humid chamber with pre-hybridization solution (50% formamide, 4× SSC, 0.5× Denhardt's, 25 mg/ml yeast tRNA, and 50% dextran sulfate). The sections were then incubated with the same pre-hybridization solution containing the sense or antisense cRNA probe for 18 h at 37°C. Next, the slides were washed twice with 2× SSC and once with 1× SCC for 1 h at room temperature, followed by washes with 0.5× SSC for 30 min at 37°C and with 0.5× SSC for 30 min at room temperature. Detection of *cch*-mRNA was performed by using an anti-digoxigenin antibody (Roche). Briefly, the samples were incubated for 4 h with an anti-digoxigenin antibody conjugated to alkaline-phosphatase (AP). After being washed, the samples were incubated by using the 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)-nitroblue tetrazolium (NBT) system (Bio-Rad) and observed with a Nikon microscope. A dark blue precipitate was considered a positive reaction. Finally, the sections were dehydrated as described above and mounted with Canada balsam. Control experiments omitting the digoxigenin-labeled probe were also performed.

Results

Immunohistochemical and cytochemical localization of hemocyanin in *Concholepas* tissues

By light microscopy, PAP immunostaining of semi-thin paraffin-embedded sections obtained from various organs of *Concholepas* females revealed an intense PAP reaction, which demonstrated the presence of CCH in blood vessels and connective tissue located in the mantle (Fig. 1b, c), hepatopancreas (Fig. 1f, g), and branchia (Fig. 1j, k). Control experiments confirmed the specificity of this reaction (Fig. 1d, h, l, respectively). Subsequently, these results were corroborated by transmission electron microscopy. We detected profuse free intact molecules that resembled hemocyanin in blood vessels and within clear vacuoles in some circulating hemocytes (Fig. 2a–f) or in cells with an appearance similar to that of rhogocyte-like cells and localized within the connective tissue (Fig. 2g–i). Because of the large size of *Concholepas* hemocyanin, which is approximately 325 Å in diameter and 392 Å in height (De Ioannes et al. 2004), and because of its peculiar structure as a hollow cylinder (Fig. 2c, insert), we were able unequivocally to identify the presence of hemocyanin molecules (Fig. 2c, f, i).

To confirm the above observations, we purified hemocytes directly from hemolymph and analyzed them by using transmission electronic microscopy, which allowed the identification of at least three types of granular hemocytes. The most abundant type of hemocyte was a phagocyte-like cell characterized by a round nucleus and numerous relatively small, dense granules in the cytoplasm; some granules contained a crystalloid material (Fig. 3a, d, g, h). A less frequent cell type contained large electron-dense granules (Fig. 3b, e, h). Moreover, we observed hemocytes that were characterized by a multilobulate nucleus and the presence of many clear vacuoles

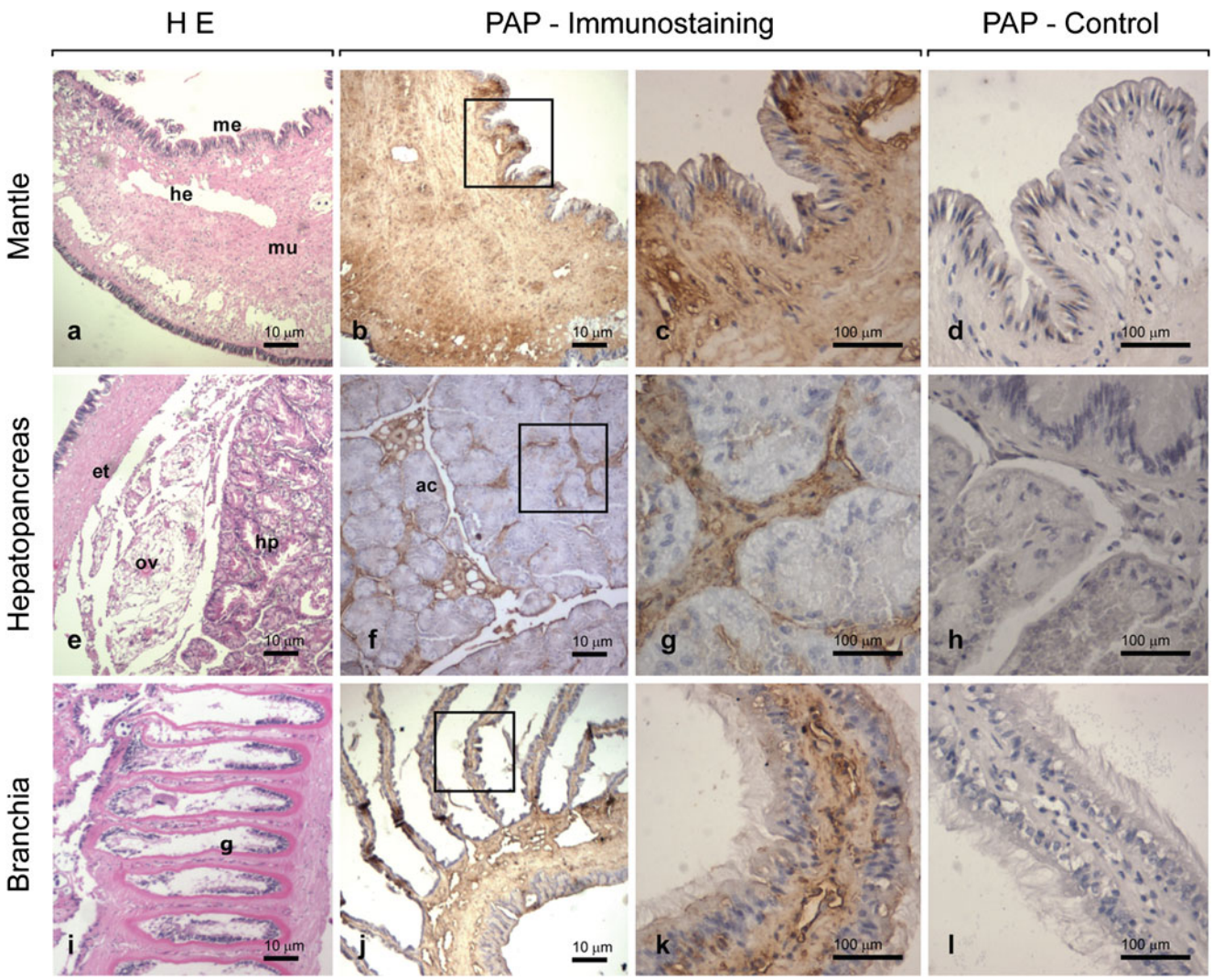


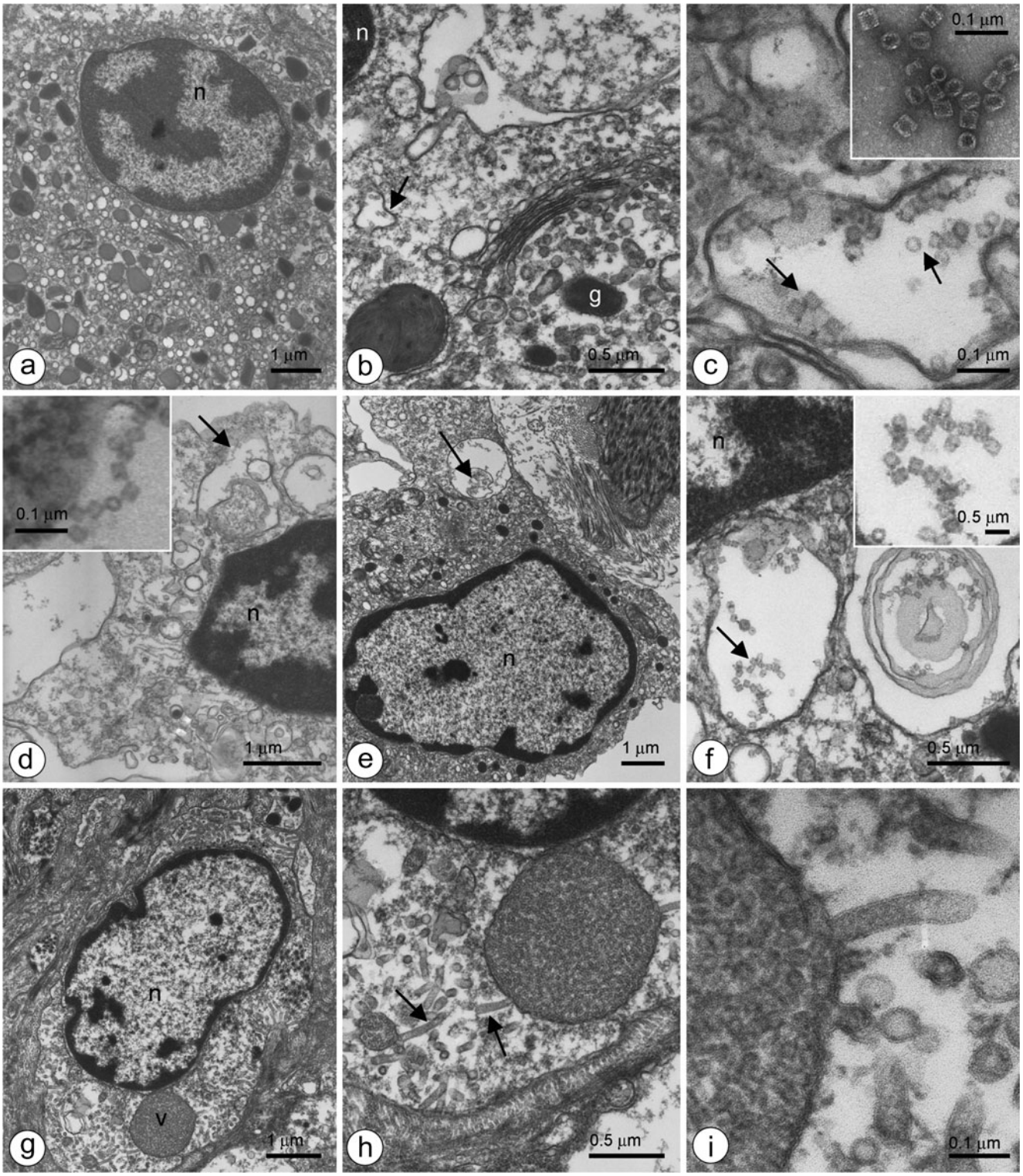
Fig. 1 Immunohistochemical localization of hemocyanin in paraffin sections of *Concholepas concholepas* tissues by using rabbit polyclonal antibodies riased against *C. concholepas* hemocyanin (CCH); light microscopy (*HE* hematoxylin and eosin; *PAP* peroxidase/anti-peroxidase). **a** Histological section of the mantle stained with HE (*me* mantle epithelium, *he* hemolymph sinus, *mu* musculature). **b** Overview of the mantle stained using the PAP method. An intense *brownish* reaction was observed in the total organ. **c** Higher magnification (*boxed area* in **b**) of the mantle epithelium showing the PAP reaction below the epithelial lining. **d** Control in which the specific antiserum was omitted. **e** Histological section of the hepatopancreatic-gonadal complex stained with HE (*et* external tunica, *ov* ovary, *hp* hepatopancreas). **f** Overview of the acinar region (*ac*) of the hepatopancreas. **g** Higher magnification (*boxed area* in **f**) of the acinus surrounded by connective tissue showing an intense PAP reaction. **h** Control in which the specific antiserum was omitted. **i** Histological section of the branchia stained with HE showing lamellae of the gills (**g**). **j** Overview of the branchia showing PAP staining. **k** Higher magnification (*boxed area* in **j**) of a branchia lamella showing the intense PAP reaction in the connective tissue below the epithelial lining. **l** Control in which the specific antiserum was omitted

containing intact hemocyanin molecules in their cytoplasm (Fig. 3c, f, i).

In addition, samples from the mantle were analyzed by colloidal gold immunostaining with rabbit polyclonal antibodies against CCH. Interestingly, we observed a high concentration of gold beads in the cytoplasm of some circulating hemocytes (Fig. 4a–c) or in cells localized in the connective tissue (Fig. 4d–f). Specifically, these beads were located inside the electron-dense granules and tubules and on the entire cell surface. No significant immuno-gold staining was detected in the control sections (Fig. 4a, d). According to the previous results, hemocytes might participate in the biosynthesis of the hemocyanin in *C. Concholepas*.

Detection of hemocyanin-mRNA in *C. concholepas* tissues

To determine the site of CCH synthesis, we employed RT-PCR to measure the transcription levels of *cch*-mRNA. Previously, we had designed degenerate primers that



amplified the conserved copper center of mollusc hemocyanins. We cloned and sequenced a segment from the *cch* cDNA and determined that its sequence corresponded to a region encoding the Cu-B site. Subsequently, we proceeded to detect *cch* transcript levels by RT-PCR in the various tissues previously analyzed by microscopic methods, including the circulating hemocytes. The results of a representative experiment are presented in Fig. 5. The RT-PCR assays revealed the presence of *cch*-mRNA only in the hepatopancreas tissue sample, and no *cch*-mRNA was detected in any amplified product from the other tissues, i.e., the mantle, branchia, or hemocytes. To verify the

◀ **Fig. 2** Morphological characterization of *Concholepas* hemocytes localized in the blood vessels and connective tissue of the mantle; transmission electron microscopy. **a** Granular hemocytes showing a round nucleus (*n*) and abundant small electron-dense granules in the cytoplasm. **b** Details of the cytoplasm showing electron-dense granules (*g*) and small clear vacuoles containing hemocyanin molecules (*arrow*). **c** Details of vacuoles are as follows: top (*circles*) and lateral (*rectangles*) views of whole hemocyanin molecules (*arrows*). *Insert* Higher magnification of hemocyanin molecules purified from the hemolymph of *C. concholepas* and negatively stained. **d**, **e** Hemocytes displaying fewer granules compared with the cell in **a** and with vacuoles containing hemocyanin molecules (*arrows*). *Insert* in **d** Higher magnification of hemocyanin molecules in the area indicated with an *arrow*. **f** Details of the vacuoles present in the above cell types showing intact hemocyanin molecules. *Insert* Higher magnification of hemocyanin molecules in the area indicated with an *arrow*. **g** A rhogocyte-like cell located in the connective tissue and characterized by the presence of dense vacuoles (*v*) containing numerous molecules that resemble hemocyanin. **h** The cytoplasm of these cells contains numerous electron-dense tubules (*arrow*). **i** Details of the vacuole shown in **h** confirming the presence of hemocyanin molecules

efficiency of the amplification reaction, we used a cDNA fragment coding for a segment of β-actin from *C. concholepas* as a positive control. As expected, the RT-PCR revealed the amplification of β-actin in every tissue analyzed.

In situ hybridization in *C. concholepas* tissues

To verify the above results, we measured the *cch*-mRNA levels by in situ hybridization on formalin-fixed paraffin-embedded tissue sections with a riboprobe specific for *cch*. We had previously synthesized a cRNA probe for this purpose by using the B-Cu site of mollusc hemocyanin as a template for the run-off technique. We labeled the *cch* cRNA anti-sense probe with digoxigenin and hybridized it to tissue sections of the mantle, branchia, and hepatopancreas. The *cch*-mRNA was detected by using an anti-digoxigenin antibody conjugated to AP, and the hybridization reaction was developed by using NBT and BCIP. A positive reaction showed a dark blue precipitate at the reaction site. By light microscopy, a specific hybridization signal was only observed in cells localized in the hepatopancreas (Fig. 6a–c). In contrast, the mantle and branchia displayed no staining (data not shown). As a negative control, we labeled the *cch*-cRNA sense probe under identical conditions (Fig. 6d). In conclusion, the results obtained by using the above-described molecular genetic assays confirmed that the hepatopancreas tissue was the location of CCH synthesis.

Discussion

To date, few studies have investigated the biosynthesis and catabolism of hemocyanin in molluscs. In general, the main difficulty is that hemocyanin molecules are found free in the hemolymph rather than in circulating cells whose lineages can be more easily traced (van Holde and Miller 1995). Based on the increase in biomedical and biotechnological applications for these proteins, topics such as the study of their metabolism and expression are relevant. For example, it is relevant to know whether the switching of hemocyanin genes in response to different environmental changes occurs. Indeed, the latter phenomenon has been invoked to explain the inability to identify the site of KLH biosynthesis to date (Albrecht et al. 2001; Oakes et al. 2004).

In this context, our goal was to determine the organ of hemocyanin biosynthesis in the marine neogastropod *Concholepas concholepas*. To achieve this goal, we studied several tissues known to perform this function in arthropods and molluscs, by using a combination of immunological and molecular genetic methods. A problem encountered in several studies investigating this topic has been the use of immunologic methods alone, which do not discriminate whether hemocyanin is being synthesized, stored, or catabolized inside a given cell type. As previously mentioned, in arthropods, unlike molluscs, the majority of studies have identified the hepatopancreas as the organ in which hemocyanin biosynthesis occurs, and the cell types have been well-defined by light and electron microscopy (Taylor and Anstiss 1999; Burmester 2002). An example is the well-studied case of *Penaeus monodon* in which F-cells specialized for the secretion of digestive enzymes also synthesize hemocyanin (Lehnert and Johnson 2002; Wong et al. 2008). In contrast to arthropods, hemocyanin biosynthesis in molluscs takes place in diverse organs, such as the hepatopancreas and the branchial gland, and even in isolated cells that comprise the connective tissue, including roghocytes (Sminia and Boer 1973; Albrecht et al. 2001).

Our results unambiguously demonstrate that hemocyanin is synthesized in the hepatopancreas of *C. concholepas*. This organ, which has been previously described as a digestive gland (Van Weel 1974), is a complex organ that is involved in the following functions: secretion of digestive enzymes; endocytosis and intracellular digestion of food particles; storage of nutrients and calcium; detoxification functions (Giard et al. 1998; Bustamante et al. 2002). The structure of the hepatopancreas in gastropods consists of numerous tubules (acini) that surround the intestine and are separated by interacinar spaces (Gupta 1977). The histological information obtained in the present study has allowed us to identify *cch*-cRNA localized in the region around the acini by using an antisense riboprobe. However, we have not identified the specific cellular type that synthesizes CCH, because the acini comprise an epithelium of various cell types. The cell types of acini in *C.*

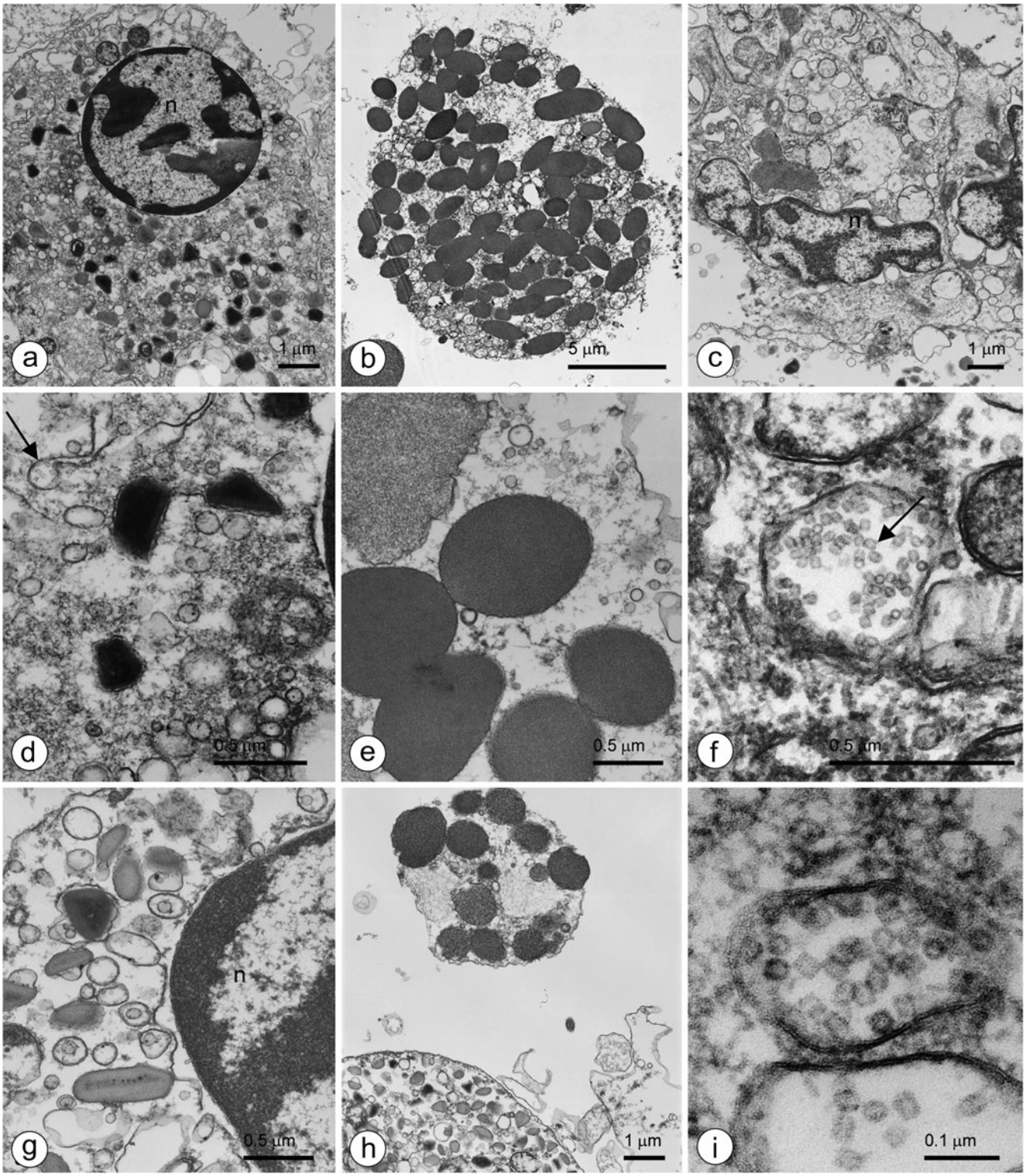


Fig. 3 Morphological characterization of *Concholepas* hemocytes isolated from hemolymph; transmission electron microscopy. **a**, **d**, **g** The most frequent cell types in the preparation of hemocytes showing a spherical nucleus (*n*) with various kinds of electron-dense granules in the cytoplasm and fewer vacuoles containing hemocyanin (*arrow* in **d**). **b**, **e** Overview and detail, respectively, of another type of hemocyte observed less frequently in the preparation, showing larger electron-dense granules in the cytoplasm. **c**, **f** Overview and detail, respectively, of hemocytes presenting an eccentric polymorphic nucleus, few granules in the cytoplasm, and huge vacuoles containing intact hemocyanin molecules (*arrow* in **f**). **h** Lower magnification view comparing the two types of granular hemocytes shown in **a**, **b**. One contains small granules (*below*), and the other exhibits large granules (*above*). **i** Hemocyanin molecules inside hemocyte vacuoles demonstrating the characteristic form of a hollow cylinder

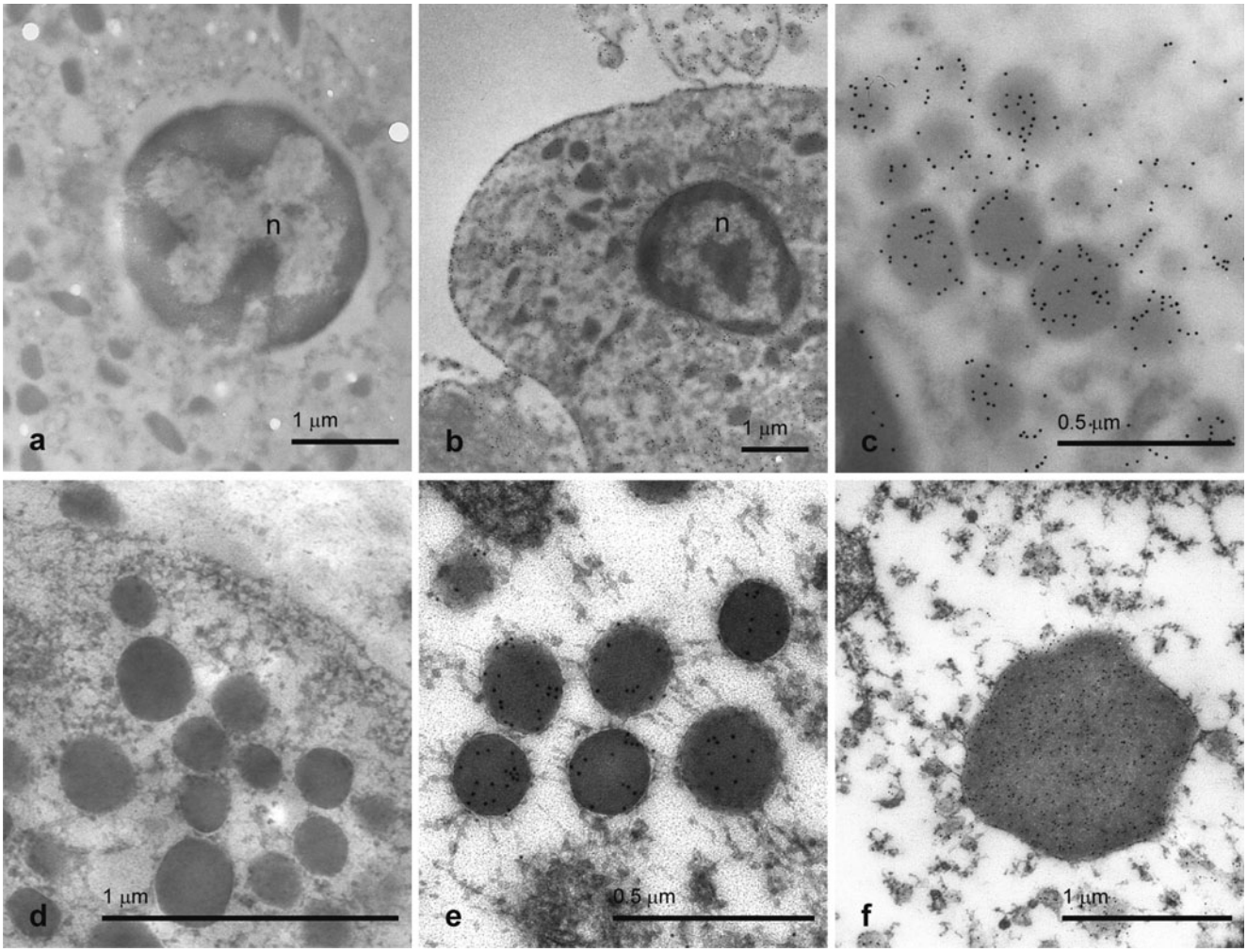


Fig. 4 Immuno-gold staining of *Concholepas* hemocytes from the mantle. **a** Control showing circulating granular hemocytes. The specific antiserum was omitted. **b** Granular hemocyte localized in the blood lacunae showing specific staining on the cell surface. **c** Higher magnification showing that the immuno-gold staining is specifically associated with electron-dense structures such as granules and tubular organelles. **d** Control showing granular hemocytes localized in the connective tissue. The specific antiserum was omitted. **e** Granular hemocyte showing numerous immunoreactive gold particles specifically located in electron-dense granules, as detailed in **f**

concholepas remain unknown, because no data are available with regard to the cytomorphology of the hepatopancreas. Further studies are needed unequivocally to identify the cells that synthesize CCH in the hepatopancreas.

Interestingly, we have detected hemocyanin in hemocytes from *C. concholepas*. However, RT-PCR analyses indicate that these cells do not play a role in the synthesis of hemocyanin in adult animals. This result is similar to that determined for arthropods. For instance, hemocyanin-mRNA has not been detected in hemocytes of the *Leiurus quinquestriatus* scorpion (Avissar et al. 1981). In contrast, hemocyanin-mRNA has not been detected in hemocytes from adult *S. officinalis*, which demonstrate positive immunostaining for an anti-hemocyanin antibody, consistent with the present results. Furthermore, hemocyanin-mRNA is detectable in embryonic hemocytes; this indicates that these cells are the site of hemocyanin biosynthesis during development (Beuerlein et al. 2004). Thus, the present study raises the following questions: what is the role of hemocyanin molecules in *Concholepas* hemocytes, and what is the role of these cells in CCH metabolism? As previously mentioned, this function has been proposed for roghocytes, a cell type that has been difficult to recognize in the connective tissue of *C. concholepas*.

The roles of hemocytes are well documented in invertebrates (Beuerlein et al. 2002b; Iwanaga and Lee 2005). These cells have a role that is identical to macrophages in mammals with respect to their phagocytic capability, defence against pathogens, and secretion of exogenous cytotoxic soluble molecules such as antimicrobial peptides into the hemolymph. In arthropods and molluscs, 99% of circulating hemocytes contain intracellu-

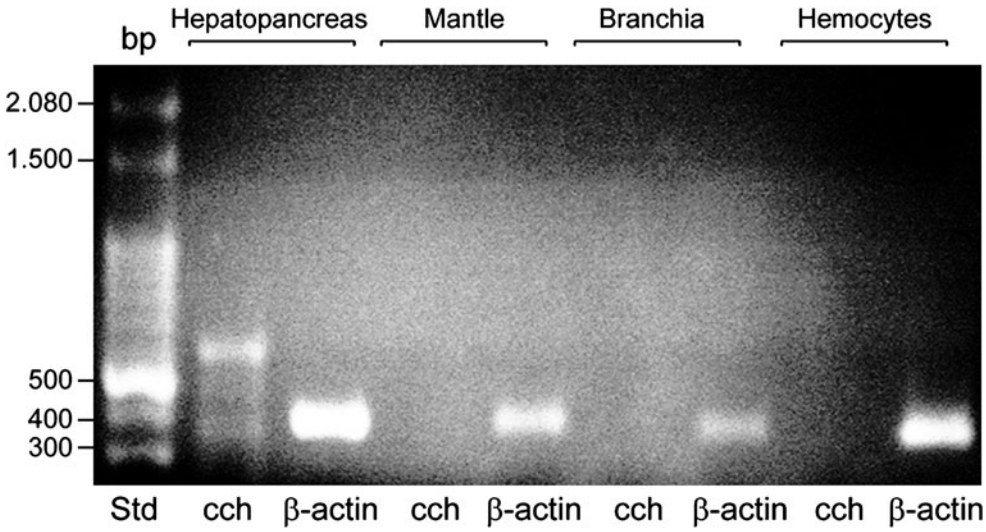


Fig. 5 Reverse transcription with polymerase chain reaction (RT-PCR) analysis of *cch*-mRNA in the hepatopancreas, mantle, branchia and hemocytes isolated from hemolymph of *C. concholepas* specimens by using *cch*-specific primers. The analysis was performed in parallel with RNA isolated from each indicated tissue. PCR products were analyzed by electrophoresis in 1.2% agarose gels prepared in 0.5× TBE (TRIS-borate, EDTA) buffer. The samples were stained with ethidium bromide and observed by using an UV transilluminator. Only the hepatopancreas demonstrated a positive RT-PCR. β-Actin primers served as a control, and PCR products were obtained from all of the tissues analyzed (*Std* standard DNA ladder size marker, *bp* base-pair

lar granules that are classified according to their size as being large or small. Our results are in agreement with this observation. The circulating and tissue-associated hemocytes correspond mostly to granular hemocytes, including large (lengths ranging between 0.8 μm to 1.1 μm) and small (lengths ranging between 0.3 μm to 0.4 μm) granular variants, which have been characterized by transmission electron microscopy. However, additional histological studies are necessary for a complete characterization, in view of the possibility that these cells correspond to intermediates during cellular differentiation (Van de Braak et al. 2002; Zhang et al. 2006).

Furthermore, in *C. concholepas*, we have identified at least two different types of hemocytes that contain hemocyanin; this suggests that they play an active role in hemocyanin metabolism, including transport or storage. We suggest that hemocytes that clearly exhibit intact hemocyanin molecules within cytoplasmic vacuoles have a role in its transport or storage (Fig. 3). In this respect, arthropods possess highly specialized cells that are designed to capture a large amount of diverse types of particulate matter, including cells that are free to migrate, and cells that are anchored to tissues such as the hepatopancreas (Johnson 1987). In addition, we have demonstrated the presence of

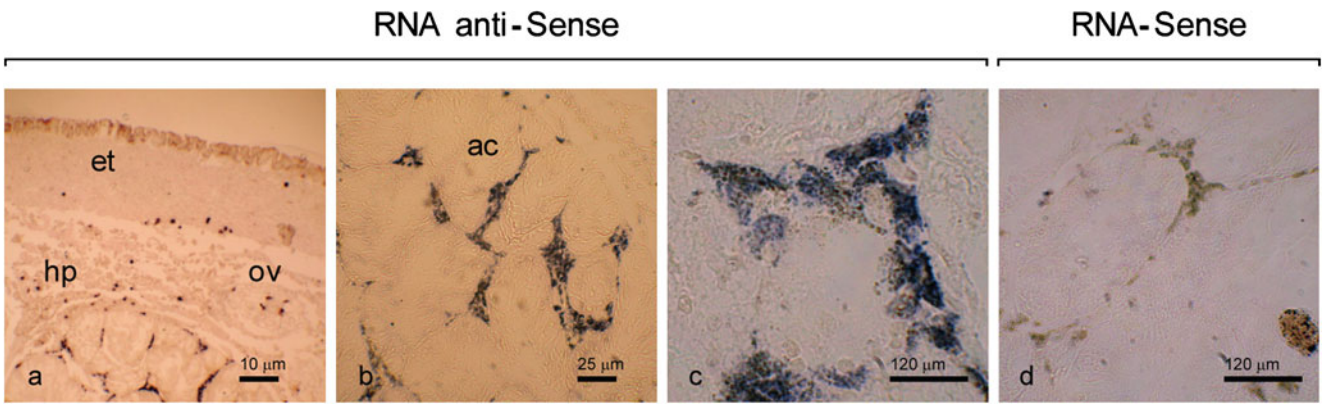


Fig. 6 Analysis of *cch*-mRNA biosynthesis in the hepatopancreas by in situ hybridization. Paraffin-embedded hepatopancreas sections were hybridized to a digoxigenin-antisense *cch* riboprobe. The assay was developed by using an alkaline-phosphatase-labeled anti-digoxigenin antibody and BCIP/NBT as a substrate. **a** Overview showing the general appearance of the hybridized hepatopancreatic-gonadal complex (*et* external tunica, *ov* ovary, *hp* hepatopancreas). **b**, **c** Details of the preparation showing the in situ hybridization signal in the hepatopancreas located in the acinus region (*ac*). **d** Control showing a section similar to that in **c** and hybridized to the sense *cch*-riboprobe

hemocyanin epitopes within electron-dense granules by colloidal gold immunostaining (Fig. 4). These results suggest that hemocytes play a role in the catabolism of hemocyanin and are also related to the generation of antimicrobial peptides. In arthropods, hemocytes have a central role in the host immune response. Therefore, the granules within these cells store antimicrobial peptides, which are released in response to microbial challenge. In some species, antimicrobial peptides are derived from hemocyanin by the processing activity of an unknown protease (Destoumieux et al. 2000; Destoumieux-Garzon et al. 2001; Lee et al. 2003; Zhang et al. 2004; Iwanaga and Lee 2005). Moreover, hemocyanins in arthropods and molluscs are processed by an endogenous seryl protease or by exogenous effectors such as detergents, thus resulting in the development of phenoloxidase activity (Cerenius and Söderhäll 2004; Garcia-Carreno, et al. 2008; Idakieva et al. 2009). In arthropods, the components of hemocytes and antimicrobial peptides induce the same activity (Nagai and Kawabata 2001; Adachi et al. 2003). These data suggest that the catabolism of hemocyanin in hemocytes is involved in the formation of secondary metabolites such as antimicrobial peptides and phenoloxidase enzymes. Indeed, Dolaska-Angelova et al. (2009) have recently reported that a glycopeptide obtained from *Rapana thomasiana* hemocyanin possesses antiviral activity.

In conclusion, we have demonstrated, for the first time, that the synthesis of *Concholepas* hemocyanins occurs in the hepatopancreas, and that hemocytes might have a role in processes other than CCH metabolism alone. These findings provide novel information that enhances our understanding of the biosynthesis and utilization of these colossal proteins in gastropod molluscs.

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