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LOCATION OF EGG-LAYING HORMONE IN REPRODUCTIVE STRUCTURES AND NEURONS OF *HALIOTIS RUBRA* (LEACH) USING ANTIBODIES RAISED AGAINST RECOMBINANT FUSION PROTEINS

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ABSTRACT Recombinant abalone egg-laying hormone was produced using a bacterial expression vector. This required directional cloning of a 108-bp abalone egg-laying hormone (aELH) gene sequence, using PCR of genomic DNA with primers incorporating restriction enzyme sites, into a pGEX-2T vector. Following transformation of the recombinant vector into *Escherichia coli*, a GST:aELH fusion protein was produced in large quantities and then purified. This fusion peptide was used to immunize mice for the production of polyclonal and monoclonal antibodies, which were subsequently tested for specificity using ELISA's and Western blots. Antisera and two IgM monoclonal antibodies were shown to react with aELH. These antibodies were used in immunocytochemistry studies of neural and gonad tissues of sexually mature female abalone. The aELH was found to be located in neurosecretory cells of cerebral and pleuro-pedal ganglia, statocysts, and trabeculae in female gonads.

KEY WORDS: egg-laying hormone, aELH, fusion protein, monoclonal antibodies, immunocytochemistry

INTRODUCTION

Immunocytochemistry has previously been used to characterize central neurons which react to antibodies raised against neuropeptides, including the α-caudodorsal cell peptide (α-CDCP) and caudodorsal cell hormone (CDCH) in *Lymnaea stagnalis*, and corresponding egg-laying hormone (ELH) in *Aplysia californica* (Van Minnen et al. 1989, Nambu & Scheller 1986). These antibodies allowed the identification of the neurosecretory cells controlling egg laying. In *Lymnaea*, the neurosecretory cells are located within the caudo dorsal cells (CDC) of the cerebral ganglia. The CDCs are arranged in two clusters in the left and right cerebral ganglia. In *Aplysia*, bag cells of the abdominal ganglion appear immunoreactive for ELH. The bag cells consist of two clusters of 250 to 400 cells located in the abdominal ganglion, near the origin of the pleurovisceral connectives.

Further investigations have indicated that the egg-laying preprohormone is relatively conserved across a wide range of molluscan classes (Nambu & Scheller 1986), resulting in the identification of additional peptides associated with neurons, which possibly control egg-laying and egg-laying behavior. Van Minnen et al (1992) have found that a number of gastropod molluscs, including *Helix aspersa*, *Biomphalaria glabrata* and *Limax maxumus*, contain immunoreactive proteins using antibodies raised against α-CDCP, a short peptide within the CDCH preprohormone. Using these antibodies, as well as antibodies against CDCH in cross-reaction studies, it has also been shown that neurons in the bivalves *Mytilus*, *Mya* and *Placopecten* also contain a similar vitellogenic factor (Croll et al. 1993). These selective immunological markers, therefore, suggest that related peptides may be involved in the egg laying of a wide range of gastropod and bivalve molluscs.

In addition, peptides immunoreactive to antisera specifically directed against CDCH, α-CDCH and β-CDCH (another short peptide in the CDCH preprohormone), have been detected in the central nervous system of the rhyonobdellid leech, *Theromyzon tessulatum* (Salzet et al. 1997). As well, it has been shown that polyclonal antisera directed against the same three peptides results in positive immunoreaction in *Sarcophaga bullata* (Diptera), *Leptinotarsa decemlineata* (Coleoptera), *Locusta migratoria* and *Periplaneta americana* (Orthoptera) (Theunis et al. 1990).

Abalones are classified as primitive gastropods and have a nervous system containing cerebral ganglia rather than a defined brain. Nerve cords and connectives lead from the cerebral ganglia, two of which join to a single pleuro-pedal ganglion. This ganglion is composed of fused pleural and pedal ganglia, both of which are elongated and flattened (Crofts 1929). A common feature of all these ganglia is the possession of an outer cortex of ganglion neurons and glial cells surrounding a central neuropril. The small neuronal cells and glial cells lie below the surface layer but do not extend into the neuropril.

Neurosecretion in the cerebral ganglia was investigated in *H. discus hannai*, in order to determine the role of hormones in the regulation of reproduction (Hahn 1994). The study showed that four types of cells exist in the cerebral ganglia, and these were defined as cell types A, B, C and D, of which only cell types A and B appear to be neurosecretory. Cell type A shows a correlation with vitellogenesis and gametogenesis in the ovary (Hahn 1994). More recently, similar neurosecretory cells of cerebral ganglia have been identified in *H. asinina* (Upatham et al. 1998). In other experiments, Yahata (1973) induced spawning of abalone by injection of homogenized pleuro-pedal and visceral ganglia of mature females, indicating the existence of an egg-laying hormone. However, injections of homogenized cerebral ganglia produced no notable change in the ovaries. Despite these cytological and physiological studies indicating the existence of sets of neurosecretory cells in the ganglia, there is no definitive evidence that peptides produced in these cells are regulating reproduction and growth.

The nucleotide sequence of an abalone ELH (aELH) has been recently obtained for *H. rubra* (Wang & Hanna 1998), and other abalone sequences (Hanna et al. 2000), all of which show high homology. Fig. 1 shows the egg-laying precursor peptide arrangements of *L. stagnalis* and *A. californica*, together with the location of the sequence encoding the aELH of *H. rubra*. The nucleotide homology of the aELH is 94% compared with the CDCH of *L. stagnalis* and 56% compared with the ELH of *A. californica*.

Despite this, very little is known about the regulation of egg-laying hormones during reproductive cycles in the genus *Haliotis*, and the sites at which aELH is expressed. Therefore, research was

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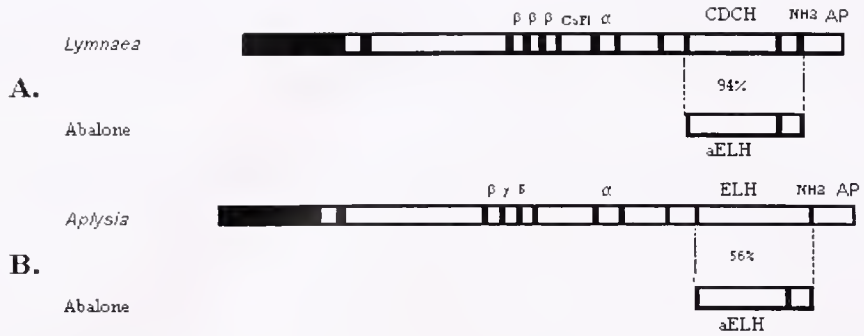

Figure 1. Comparison of *Lymnaea* CDCH (A, Vreugdenhil et al. 1988) and *Aplysia* (B, Scheller et al. 1982) egg-laying preprohormone sequences with the known abalone sequence. NH2 represents a potential amidation signal. Percentage nucleotide homology between corresponding regions are indicated. Positions of potential peptides are indicated by β_{1-3}-CDCP/β-BCP, γ-BCP, α-CDCP/αBCP, δ-BCP, calfluxin (CaFl) and an acidic peptide (AP). Known or potential cleavage sites are shown with black bars, and the larger black regions (left) represents signal sequences at the 5′ region.

carried out to develop antibodies for subsequent use in immunological techniques to locate cells producing egg-laying hormone within abalone neural and reproductive organs.

MATERIALS AND METHODS

Recombinant Protein Expression and Purification

Recombinant aELH was expressed in large quantities and purified by the use of a pGEX-2T (Amersham) expression vector in which the 108-bp egg-laying hormone sequence of *H rubra* had been inserted (Wang & Hanna 1998). This recombinant plasmid was transformed into *E. coli* competent cells and colonies selected on LB medium containing ampicillin. A 10-mL bottle of prewarmed 2× LB/Amp medium was loop inoculated with cells from one colony, and incubated at 37°C with shaking overnight. This culture was transferred into 1 L of the same medium and grown at 37°C with shaking until A_{600} reached 0.5-0.2. Bacterial fusion proteins were induced by adding 0.5 mL of 100mM IPTG with incubation for another three to five hours. The culture was centrifuged at 8,000 rpm for 5 min at 4°C (Beckman, JA-20) in 40-mL conical flasks. The supernatants were discarded and the tubes placed on ice. The pellets were resuspended in ice-cold PBS and the cells sonicated for five 30-second intervals on ice, ensuring that no frothing occurred. Immediately following this, 10% Triton X-100 was added to make final concentrations of 1% and the solutions were then incubated for 30 minutes with gentle mixing on ice to aid in solubilization of fusion protein. The suspensions were centrifuged at 10,000 rpm for 10 min at 4°C and supernatants transferred to 50-mL centrifuge tubes. Glutathione sepharose 4B beads were mixed with each 100 mL of sonicate and the contents left to stand for one hour. The glutathione sepharose 4B beads with fusion protein were sedimented by centrifugation and the supernatant collected for later reference. Ice-cold PBS was added and beads washed three times by repeated centrifugations. The fusion protein was then eluted with glutathione elution buffer, and analysis of fusion protein was performed by SDS-PAGE and Coomassie blue staining.

Production of Antibodies

Antigens used in primary immunizations were prepared by mixing 50 μg of the fusion protein with an equal volume of Freund's complete adjuvant (Sigma). BALB/c mice were then immunized intraperitoneally. Subsequent immunizations were performed each wk, for 3 wks, using the same dose of fusion protein but mixed with Freund's incomplete adjuvant (Sigma).

Polyclonal antisera were obtained by tail-bleeding mice and stored for use when ELISA end-point titres of >10,000 were obtained. For immunocytochemical studies, antisera were diluted 1/500 to 1/5000. Monoclonal antibodies were prepared four days after the last injection of immune mice, using standard techniques (Goding 1987). The mice were killed by cervical dislocation and the spleens removed aseptically. Spleen cells were fused with Sp2/0-Ag-14 myeloma cells using PEG 4000. The supernatants of the hybridomas surviving the selective medium consisting of RPMI 1640, bovine fetal calf serum and HAT, were tested for the presence of specific antibodies by means of ELISA. Antibodies secreted from positive clones were isotyped and the cells re-cloned twice to ensure monoclonality.

Quantification and Specificity of Antibodies in ELISA

ELISAs were undertaken to determine the titre of anti-aELH sera obtained from mice and for screening hybridomas and specificity of reaction with antigen. Antigen concentrations, which were calibrated to 20 μg/ml, consisted of expressed GST-aELH fusion protein or expressed GST.

The wells of a microtitre plate (Bio-Services) were coated with 50 μL of antigen solution, which was allowed to adsorb to the plate at 4°C overnight. Unoccupied binding sites on the plates were blocked with 1% BSA in coating buffer for one hour at 37°C. The plates were then washed three times in washing buffer and incubated with 50 μL of polyclonal antiserum diluted to 1:50, 1:100, 1:500, 1:1000, 1:5000 and 1:10000, in incubation buffer at 37°C for one hour. Supernatants of hybridomas were substituted for the antisera in additional tests. For the detection of bound antibody, plates were washed three times in washing buffer and incubated for one hour at 37°C with 50 μL per well of a secondary alkaline phosphatase labeled antibody (Sigma) diluted 1000-fold in incubation buffer. Plates were then washed three times in washing buffer and reacted with a 50 μg/mL solution of the enzyme substrate. After 100 min of color development, the plates were read on an automated ELISA plate reader (Titertek Multiskan MCC/340) at 405 nm.

Dissection of Abalone Tissues

Live female *H. rubra* (Leach) with mature gonads were collected under a research permit (97/R/049A), during the months

September to November. If not used immediately they were transferred to an abalone aquaculture facility where they were kept for no more than 5 days under a 12-h light/12-h dark cycle. Animals were removed from the shell prior to dissection.

To remove the left and right cerebral ganglia (cg), a blunt probe was used to separate the tissues from each side of the head. The cg with two nerves protruding was then detached. Next, the pleuropedal ganglia (ppg) and statocysts were exposed by removing all organs in the head cavity with a scalpel and then removed from within the muscle tissue. A piece of female gonad was also removed from the conical end of the organ.

Fixation and Sectioning of Tissues

After dissecting out the ganglia and pieces of gonad, they were fixed in freshly prepared 4% paraformaldehyde at 4°C for 24 h. They were then transferred to phosphate buffer, and stored at 4°C before further processing.

Prior to cryosectioning, tissues were transferred to 30% sucrose in phosphate buffer and stored overnight at 4°C. The preparations were then frozen in TissueTek (Bayer Diagnostics) in the cryostat at −20°C and subsequently sectioned at 6 μm. Alternate sections were placed on separate slides so that different antibodies or control procedures could be compared. Slides were then stored at −80°C, until required.

Western Blots

Standard SDS-PAGE of expressed GST and GST-aELH were carried out prior to Western transfer of the proteins onto 0.45 μm nitrocellulose membranes. The membranes were then reacted with antisera or mAbs to determine the sizes of antigenic proteins.

Fresh tissues of cg, ppg and gonad were used to prepare total protein extracts using TriPure isolation reagent (Roche), according to the manufacturer's instructions. These extracts were separated on SDS-PAGE. Proteins were transferred, as before, and probed with antisera or mAbs.

Immunocytochemistry

A DAKO LSAB2 (streptavidin-biotin labeled) kit was utilized to identify the location of aELH in sections. Initially, sections were heated in buffered citrate (pH 6.0) to enable antigen retrieval. To prevent endogenous peroxidase activity, the slides were treated with 3% hydrogen peroxide, after which, primary antibody (diluted polyclonal or monoclonal in ascites fluid) was added and the slides incubated for 1 h at RT. After washing with PBS, biotinylated anti-mouse immunoglobulins were added and the slides incubated for 30 min at RT. After a brief washing, two drops of streptavidin peroxidase were added and another incubation carried out at RT for 20 min. During this step, a fresh solution of 3-amino-9-ethylcarbazole (AEC) chromogen was prepared. The chromogen was added after another washing and a further incubation at RT for 3 to 5 min performed, before being examined for color development. When appropriate, color development was stopped by washing the slides in distilled water, a counterstain in Mayer's haematoxylin performed, and the slides washed again and before mounting in Faramount aqueous mounting solution (DAKO). Digital images were then taken using a Zeiss Axioskop MC 80.

Immunofluorescence was performed on stored frozen sections. The sections were allowed to thaw at RT for 30 min before blocking, first in 1% glycine for 30 min, then in 4% BSA for 30 min. They were rinsed three times in PBS, and then incubated for 1 h at room temperature in primary antibody (i.e. polyclonal or monoclonal). After three rinses in PBS, they were incubated in FITC-labeled goat anti-mouse secondary antibody (Silenus) in the dark at RT for 1 h, and then rinsed three more times in PBS, before mounting in FITC mounting solution. Sections were viewed under blue light with a Zeiss Axioskop MC 80 microscope and images recorded.

Negative control procedures included the omission of primary antibodies and preadsorbing the anti-aELH antibody with recombinant aELH.

RESULTS

Monoclonal Antibodies

Table 1 shows the properties of five monoclonal antibodies (mAbs) that were produced with medium to strong reactions in ELISA and immunofluorescence testing. MAbs with weak reactions were discarded. Three mAbs (i.e. F61P3E3, F62P1A1 and F62P6D4) reacted with the GST component of expressed GST-aELH fusion protein as well as expressed GST. The other two mAbs (i.e. F61P1A5 and F62P4C1) reacted with the aELH component of expressed GST-aELH fusion protein. It was unusual that most of the mAbs were IgM as a period of four days from the last immunization of peptide to the production of mAbs would have allowed for class-switching of cells from IgM to IgG's.

Specificities of mAbs were also confirmed using Western blots of expressed GST and GST-aELH fusion proteins (data not shown).

Western Blots of aELH in Abalone Tissues

The results of Western blots of cg, ppg and mature female gonad proteins are shown in Fig. 2. There were no bands at approximately 4 kDa, the estimated size of aELH (36 amino acid residues deduced from a 108 nucleotide sequence), in any of the four experimental lanes. This absence may be attributed to the small size of the aELH (i.e. 4 kDa) and relatively small amounts present, compared with the total protein present. However, the cg showed 6 bands of sizes ranging from 24 to >77 kDa, the ppg 3 bands of which two were between 34 and 48 kDa and the other >77 kDa, and the gonad showed two bands between 34 and 40 kDa. The presence of these multi-bands indicates the different processing of preprohormones in the tissues and/or the presence of multigene products.

TABLE 1.

Monoclonal antibodies produced and their properties

Monoclonal Antibody	Isotype	Specificity in ELISA	Reactivity using Immunofluorescence
F61P3E3	IgM	GST	+++
F62P1A1	IgM	GST	++
F62P6D4	IgG_1	GST	+++
F61P1A5	IgM	aELH	++
F62P4C1	IgM	aELH	++

+++, strong reaction; ++, medium reaction; +, weak reaction (mAb discarded)

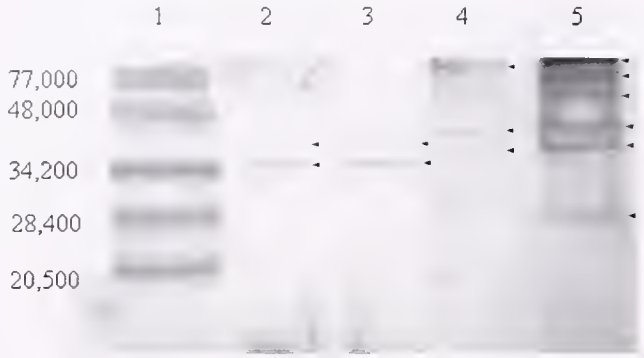

Figure 2. Western blots of proteins from *H. rubra* tissues using anti-aELH antibodies. Lanes: 1, pre-stained molecular weight markers (Bio-Rad); 2 & 3 mature female gonad; 4, pleuro-pedal ganglia; 5, cerebral ganglia.

Immunocytochemistry

A brick-red precipitate of the immunological test, together with a blue haemotoxylin counter stain, gave clear indications of antigen location. We had initially used 3,3′ diamino-benzidine tetrahydrochloride (DAB) in the final reaction, to give a brown precipitate, but found that there were endogenous brown pigments in control tissues (data not shown). This made it difficult to determine the location of the immune reaction, so this method was substituted by the AEC substrate to give a clearer result.

Consequently, immunopositive reactions were observed in cg, ppg, and mature female gonads (Fig. 3). However, there were marked differences in each of the structures. The cells reacting in the cg were equivalent to the NS1 neurosecretory cells observed by Upatham et al. (1998). In the ppg, peripheral neurosecretory cells have also been identified by the same group (unpublished), and our current work showed these cells immunoreactive. Immunoreactive material was also observed around the statocysts. In the gonads, a small number of cells spread throughout the trabeculae showed strong immunoreactions.

The results obtained using immunofluorescence location of aELH were the same as for immunoenzyme staining (Fig. 3).

DISCUSSION

Production of immunocytochemical probes has relied extensively on the use of native protein to provide specific polyclonal and monoclonal antibodies. However, there has been a transgression toward the use of synthetic or recombinantly expressed proteins, to facilitate antibody probe production. The advantage of these proteins is three-fold: large amounts of protein can be produced; only partial sequence data needs to be obtained; and laborious protein purification techniques can be avoided. In this study, the aELH sequence data obtained (Wang & Hanna 1998) was used for the *in vitro* expression and subsequent immunization of experimental animals. Antibodies were then produced that with immunoreactivity toward the recombinant protein in ELISA. However, further analyses were required to determine immunoreactivity to native protein *in vivo*, and this was ratified by immunocytochemical studies. This was not unexpected as previous studies have shown that antibodies raised against native ELH protein of *Aplysia* is immunoreactive against a synthetic ELH peptide.

In addition to ELISA, further specificity of the monoclonal and polyclonal antibody immunoreactivity to aELH was achieved by Western blots. These confirmed that the antibody probes were reactive to a protein extracts from the central nervous system and the gonad. The antibodies against aELH recognized proteins of various sizes within gonad, cerebral and pleuro-pedal ganglia extracts. This indicates that there are a number of polypeptides of different sizes, each containing the aELH peptide component. These bands probably represent differential cell processing of the polypeptides, typical of that found in *A. californica* (Fisher et al. 1988). They showed by Western blot analysis that antibodies recognized small final-product peptides, intermediates in the processing pathway which contain the sequence used as the immunogen, and also the large prohormone. However, the multi-band data may also be the result of the presence of a multigene aELH family, the genes of which are expressed in different tissues. We have preliminary data (unpublished) indicating that abalone have several genes encoding aELH. Our data is consistent with the findings of Nambu and Scheller (1986), in their studies of *Alysia* genomes. They used Southern blotting, gene cloning and immunocytochemical techniques to identify and characterize ELH related genes. Overall, there is a need for a substantial investigation into the number of aELH related genes in the abalone genome, the cellular expression of these genes, and the processing of preprohormones in each tissue to give immunoreactive proteins of various sizes.

In vivo expression of the female *H. rubra* aELH was shown in our studies of sections of abalone tissues, using monoclonal and polyclonal antibodies. The aELH appeared to be localized to the NS1 cells within the cerebral, and were also found in pleuro-pedal ganglia, statocysts, and to the trabeculae of the gonad. Our data supports the previous research by Hahn (1994) and Upatham et al. (1998), in which they showed that differential staining could identify neurosecretory cell types in the cerebral ganglia. In addition, the cerebral ganglia of other molluscan species are known to be a major site of ELH-like expression (Croll et al. 1993, Van Minnen et al. 1992).

Immunoreactivity within the pleuro-pedal ganglia was not only localized to the cells of the periphery, but also the statocyst margins and interior. The statocyst is a chambered sense organ containing granules to sense the direction of gravity.

Despite extensive research being conducted on neuropeptide immunoreactivity within the central nervous system, little research has focused on the reproductive tissue. Our study, however, shows aELH located in the trabeculae of the mature pre-spawned female gonad. It was only located within certain cells, which may function to store aELH with the maturing gonad. It is postulated that a stimulus may act to release the hormone that acts directly on the smooth muscle of the gonad to release ova. Evidence from *Drosophila* shows that when a male mates and releases sperm, he also deposits an egg-laying hormone that induces egg-laying in the female (Park & Wolfner 1995). This hormone is related to the ones found in gastropods and other invertebrates.

The large amount of aELH expressed within the reproductively pre-spawned mature female may be a function of the gastropod requirement to release high concentrations of hormone into the hemolymph. It is known in molluscs that hormone release areas are high in number (Joosse 1979).

The antibodies we have produced can now be extended to further studies. They could be used to determine site-specific expression of aELH during the reproductive cycle *of H. rubra*, and other abalone species. Preliminary tests have indicated that they do react with the aELH in *H. asinina*. It would be interesting to determine if bioassays of gonads could quantify the presence aELH sufficiently to indicate spawning readiness. We are already doing bioassays with the aELH to induce spawning.

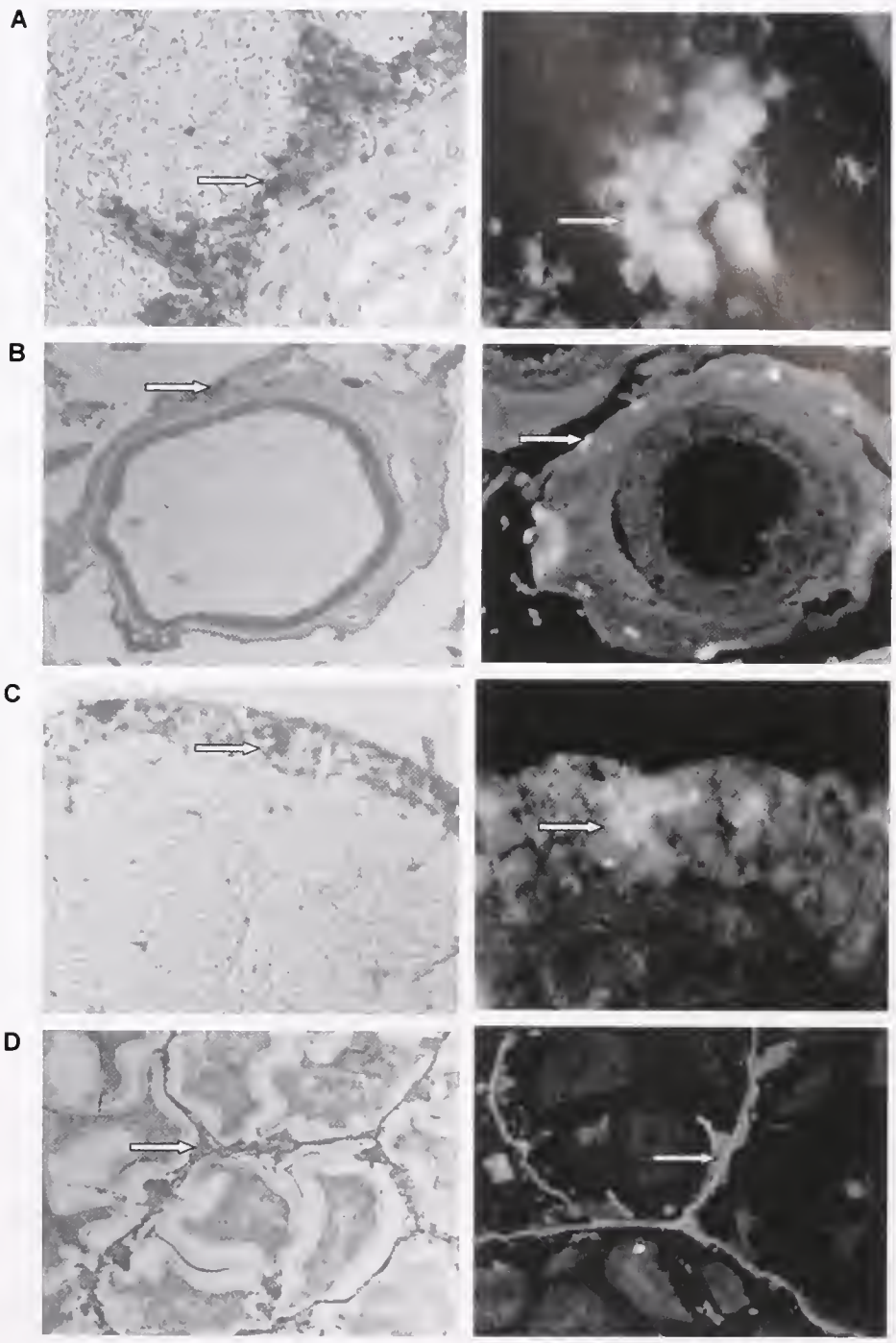

Figure 3. Localization of aELH in tissues of *H. rubra* using immunocytochemistry staining. Left: Tissues incubated with murine anti-aELH prior to detection using a DAKO LSAB2 kit to produce brick-red precipitate. Right: Tissues incubated with murine anti-aELH prior to detection using secondary incubation with FITC-conjugated goat anti-murine Ig to produce a green immunofluorescence under UV. A, immunoreactivity of aELH in cerebral ganglia; B, immunoreactivity of aELH in cells of a pleuro-pedal ganglia; C, immunopositive material around statocysts; D, immunopositive cells in the trabeculae of mature female gonads.

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