The cement apparatus of larval and adult *Pomphorhynchus laevis* (Acanthocephala: Palaeacanthocephala)

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SUMMARY

Light and electron microscopy were used to study the ultrastructure of the cement apparatus, namely cement glands and cement ducts of mature specimens of the acanthocephalan parasite *Pomphorhynchus laevis* Müller, 1776, recovered from the digestive tracts of fish *Leuciscus cephalus* Risso, 1826. In addition, the cement glands of immature *P. laevis* found within the body cavity of the fish *Alburnus alburnus alborella* De Filippi, 1844 were examined. In a mature male of *P. laevis* the 6 cement glands are rounded to oval in shape and each of them has an outer cytoplasmic layer containing nuclei and surrounding a space for storage of the cement material within the gland. The nuclei have an irregular outline and the cytoplasm of the cells contains round, membrane-bound secretory granules approximately 1 μm in diameter. Nuclei surrounded by secretory granules were present inside the gland lumen. Within the gland ducts of mature males, granules were present within the wall thickness and, inside the luminal area, mitochondria were encountered. In contrast, within the cement glands of immature *P. laevis* there were no secretory granules and the chromatin of the nuclei appeared condensed. The nature of the secretory product of the cement glands was investigated with histological and electrophoretic methods. A protein with molecular weight of 23 kDa was recorded as a major component of cement.

Key words: acanthocephalan, cement apparatus, fine structure, secretory product.

INTRODUCTION

The cement glands are among the most conspicuous and distinctive elements in the genital apparatus of male acanthocephalans. The glands and their products have considerable importance in the reproductive process. They vary in structure, shape and number and are important elements in taxonomic descriptions of acanthocephalans (Van Cleave, 1949*a*; Yamaguti, 1963; Bullock, 1969). Furthermore, they usually conform to 1 of the 3 basic arrangements suggested by Van Cleave (1949*a*). Van Cleave's description of the types of cement glands in the 3 classes of Acanthocephala does not explicitly describe the structures of all known species. In fact, changes in the number and structural organization of cement glands in a few species were reported by Van Cleave (1949*b*) for *Echinorhynchus gadi*, by Whitfield (1969) for *Polymorphus minutus*, and by Amin (1975*a*, *b*) for *Acanthocephalus parksidei*.

The glands produce secretions which function in cementing the posterior ends of the couple together during copulation; afterwards, the secretions of the cement glands seal the female gonopore with a cap. Abele & Gilchrist (1977) have proposed that the capping behaviour evolved in response to sexual selection and functions in preventing subsequent insemination. The occurrence of misplaced copulation caps on other parts of the female body (Nicholas & Hynes, 1958) as well as caps on the bodies of male individuals was reported by Abele & Gilchrist (1977) and Parshad & Crompton (1981).

To date, there have been limited observations made on the nature of the cement gland secretions (see Miller & Dunagan, 1985). Nonetheless, staining properties and the occurrence of well-developed rough endoplasmic reticulum within the cytoplasm of glandular cells indicate that protein is a component of the cement (Haley & Bullock, 1952; Parshad & Crompton, 1981). In this study, our goal was to gain information on the nature of the cement; therefore, besides histological staining, electrophoretic separation of proteins present in specimens *in toto* as well as in isolated cement glands of *Pomphorhynchus laevis* was performed. According to Meyer (1933), acanthocephalan cement glands are of the exocrine, namely holocrine, type. In the present study we provide data regarding the organization of exocrine cement glands in *P. laevis*. Very little work has been carried out on the ultrastructure of the cement apparatus of Acanthocephala; the only 2 electron micrographs in publication appeared in a paper by Asaolu (1981). Recently we surveyed the fine structure of cement glands of 4 species of acanthocephalans belonging to 4 classes of this phylum (B. S. Dezfuli, unpublished). In the case of *P. laevis*

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in a preliminary study with well-known histological methods we found that the cement in the gland and duct stained blue with Heidenhain's iron haematoxylin, but there was no reaction with Periodic acid-Schiff (PAS).

Here, we provide data describing (a) the ultrastructure of cement glands and the cement duct of mature *P. laevis* as well as glands of immature specimens, (b) the likely formation of granules within the gland cells, (c) the secretory function of the cement duct and (d) the nature of the cement.

MATERIALS AND METHODS

Specimens of chub, *Leuciscus cephalus* (total length ranging from 250 to 420 mm) and *Alburnus alburnus alborella* (total length ranging from 100 to 140 mm) were sampled by angling at Carturo, north of Padua, in the River Brenta. The fish were taken to the laboratory; after dissection of the host, the whole digestive tract was removed from each chub and searched for acanthocephalans; the body cavity and viscera of *A. alburnus alborella* were examined for extraintestinal helminths. Cystacanths of *P. laevis* were recovered from the haemocoel of amphipods *Echinogammarus stammeri* from the River Brenta.

Transmission electron microscopy (TEM)

Mature male specimens of *Pomphorhynchus laevis* were recovered from the alimentary canal of *L. cephalus*, while immature individuals were isolated from mesenteries on the outer surface of *A. alburnus alborella* gut walls. Ten mature and 8 immature worms were selected and dissected, and the whole genital apparatus (testes, vasa deferentia, seminal vesicles, cement glands and bursa) processed as follows. The male reproductive organs were fixed in 2% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·2, post-fixed in 1% osmium tetroxide in the same buffer for 1·5 h, dehydrated in a graded ethanol series and embedded in an Epon-Araldite mixture. Semi-thin sections of the cement glands and gland duct were cut on a Reichert Om2 ultramicrotome with glass knives, and stained with azure-A methylene blue. Ultra-thin sections were obtained with a Reichert Ultracut ultramicrotome stained with uranyl acetate and lead citrate and observed with a Zeiss EM9. Light micrographs were obtained with a Leitz photomicroscope.

Histological staining

Cement glands of adult *P. laevis* were fixed in Bouin's fluid, embedded in plastic paraffin, and serially sectioned at a thickness of 7 μm. Two staining methods were used, part of the material was stained with Heidenhain's iron haematoxylin and part with PAS.

Analysis of protein contents of Pomphorhynchus laevis

Electrophoretic analysis of *P. laevis* proteins was performed by SDS–PAGE (Laemmli, 1970) using the Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Milan, Italy) according to the manufacturer's instructions. Briefly, adult males and females, male cystacanths, and different amounts of isolated cement glands were solubilized in SDS lysis buffer (62·5 mM Tris–HCl, pH 6·8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0·1% bromophenol blue), sonicated for 15 sec, boiled for 5 min and centrifuged at 13000 ***g*** for 3 min. Supernatants and molecular weight markers (High Molecular Standard Mixture plus Dalton Mark VII-L, Sigma Chemical Co. St Louis, USA) were applied to a 10% continuous polyacrylamide gel and electrophoresized at 200 volts, until the tracking dye reached the bottom of the gel. Proteins were visualized by Coomassie R250 stain (Harlow & Lane, 1988).

RESULTS

Fine structure of the cement gland of immature P. laevis

The cement glands of male cystacanths of *P. laevis* recovered from extraintestinal sites in *A. alburnus alborella* were enclosed in the ligament sac (Fig. 1A). In semi-thin sections, the glands had a round-oval shape with no lumen and appeared as compact structures with many nuclei with no plasmalemma between them (Fig. 1A). In the same glands observed with electron microscopy, the nuclei were characterized by many randomly distributed zones of heterochromatin (Fig. 1B and C). Around the nuclei, many round and elongated mitochondria with lamellar cristae and some membranous translucid vesicles were observed (Fig. 1C). The cytoplasm adjacent to nuclei appeared more electron transparent (Fig. 1C) than the rest of the cytoplasm, which was filled by a high number of ribosomes.

Gross morphology of the cement glands of mature P. laevis

The 6 cement glands of *P. laevis* are arranged in 3 pairs and situated posterior to the testes (Fig. 2A). In semi-thin sections, the cement substance within the gland and duct lumen stains deep blue with azure-A methylene blue. Thin sections through the cement gland show the occurrence of 2 different parts within the gland: an outer 'cytoplasmic layer' and a luminal area ('vesicular region', see Van

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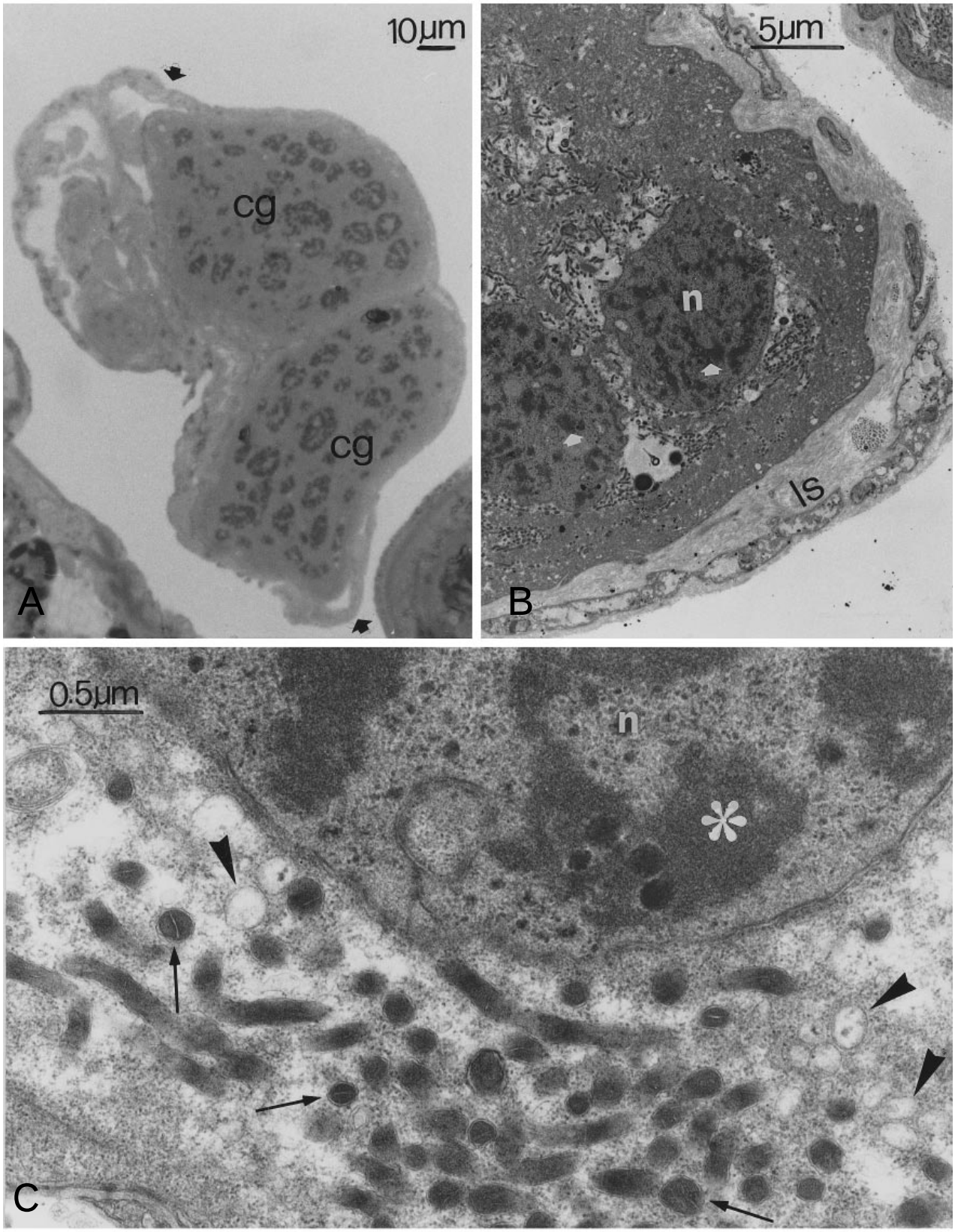

Fig. 1. (A) Semithin section of 2 cement glands from an extraintestinal *Pomphorhynchus laevis* enclosed in the ligament sac (arrows). cg, Cement gland. (B) Transmission electron micrograph of cross-section of a cement gland of an immature *P. laevis*. Note the 2 nuclei in periphery of gland, translucid cytoplasm around the nucleus and distribution of heterochromatin (white arrows). ls, Ligament sac; n, nucleus. (C) Electron micrograph showing the occurrence of mitochondria (arrows) and translucent vesicles (arrow heads) near the nucleus. Zone of heterochromatin (asterisk) is visible. *n*, Nucleus.

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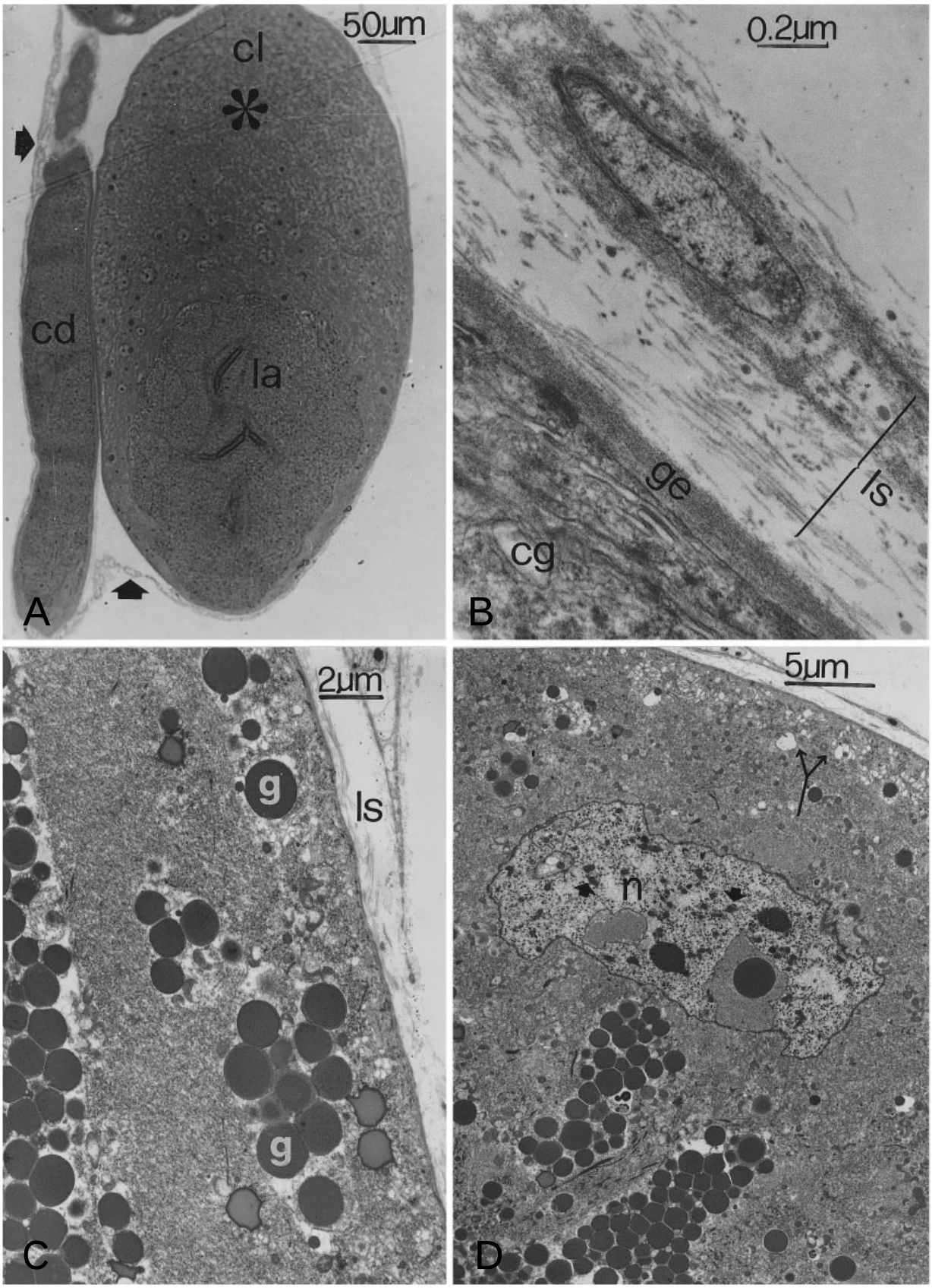

Fig. 2. (A) Semithin section of longitudinal view of mature *Pomphorhynchus laevis* cement gland (asterisk) and cement duct, both enclosed with ligament sac (arrows). cd, Cement duct; cl, cytoplasmic layer; la, luminal area. (B) High magnification of periphery of cement gland and ligament sac, cg, Cement gland; ge, gland envelope; ls, ligament sac. (C) Portion of cytoplasmic layer of a cement gland with the secretory granules. g, Granules; ls, ligament sac. (D) Micrograph of the transverse section of cytoplasmic layer, note the distribution of heterochromatin (arrows) within the nucleus and translucid vesicles (thin arrows) in periphery of gland. *n*, Nucleus.

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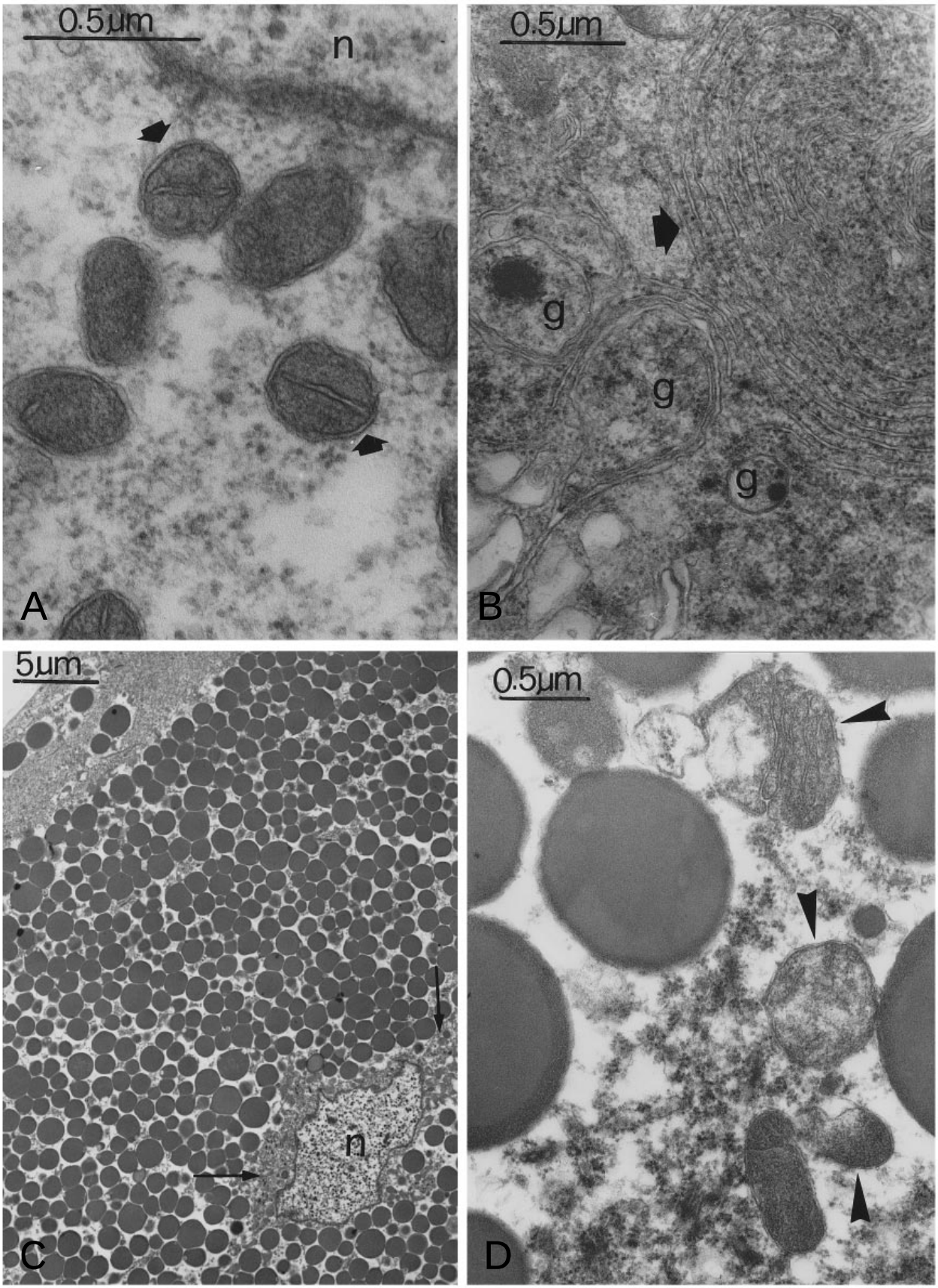

Fig. 3. (A) High magnification of cluster of mitochondria (arrows) close to the nucleus within the cytoplasmic layer. *n*, Nucleus. (B) Secretory granules in close contact with rough endoplasmic reticulum (RER, arrow). g, Granules. (C) Low magnification of a portion of cement gland, note the occurrence of nucleus surrounded by residue of cytoplasm (arrows) within the luminal area of gland. *n*, Nucleus. (D) Micrograph shows the middle portion of the gland lumen, the presence of degenerated mitochondria (arrows) can be seen.

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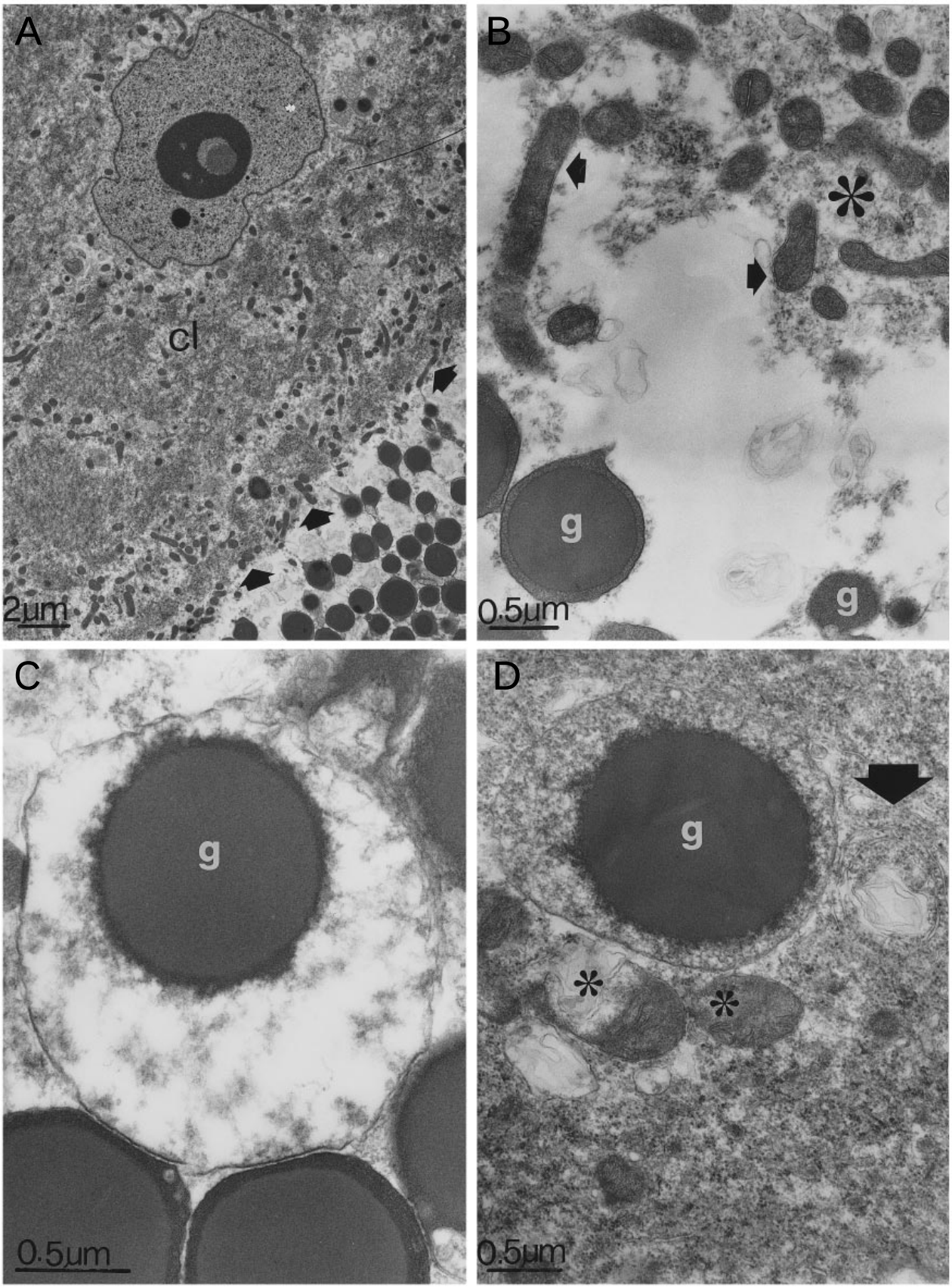

Fig. 4. (A) Luminal side of cytoplasmic layer, note the presence of mitochondria (arrows) in periphery of lumen. cl, Cytoplasmic layer. (B) High magnification shows the occurrence of mitochondria (arrows), residue of cytoplasm (asterisk) and secretory granules within the periphery of the lumen of the cement gland. g, Granules. (C) Secretory granule in formation, g, Granule. (D) Fine granular substance filled the white space between the electron-opaque inclusion and the envelope, note the relationship between RER (arrow) and granule. g, Granule; asterisks, mitochondria.

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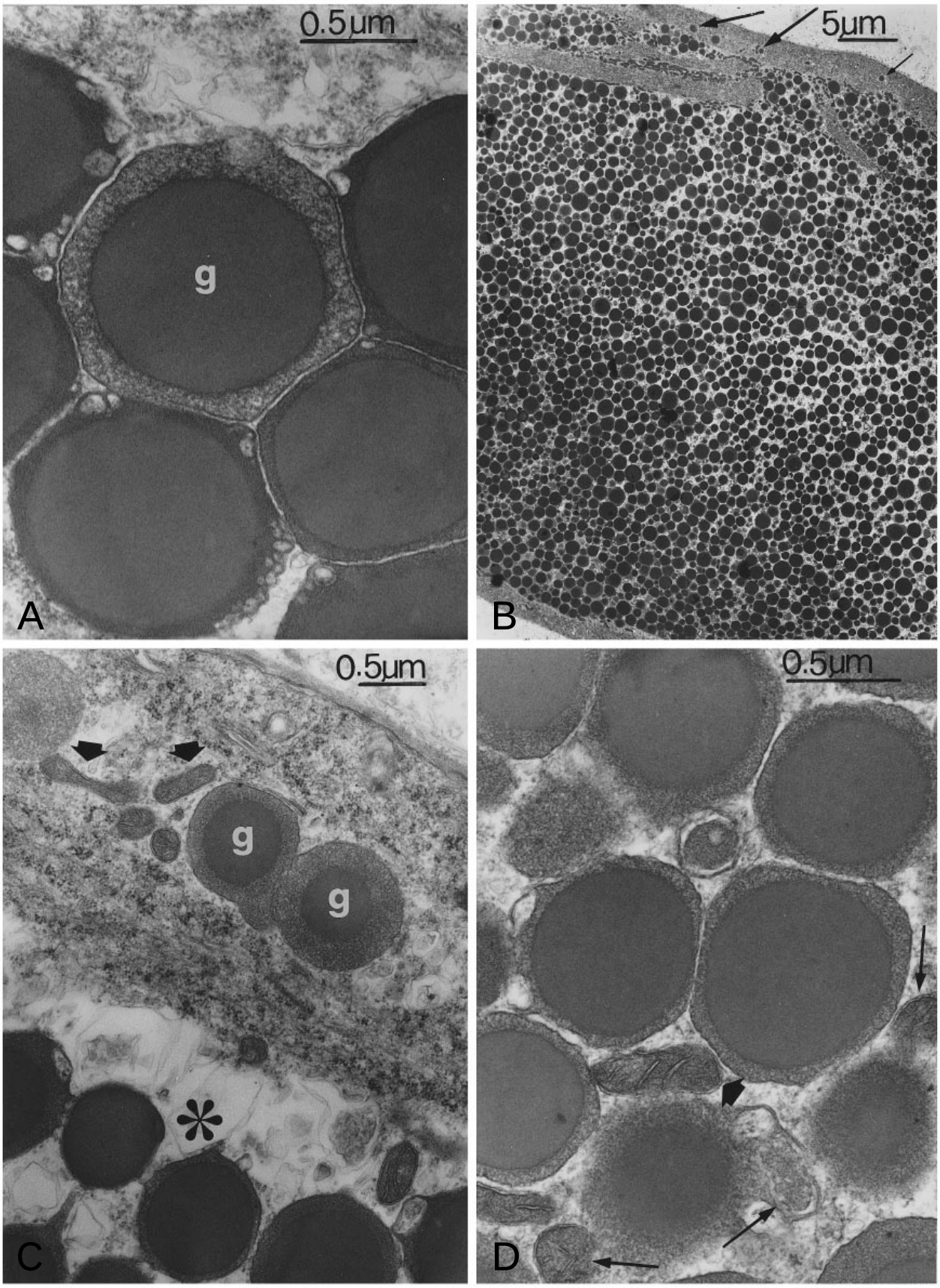

Fig. 5. (A) Micrograph shows the presence of 5 mature granules around a granule in maturation within the thickness of cytoplasmic layer. g, Granule. (B) Low magnification of longitudinal view of a cement duct, note the occurrence of secretory granules (arrows) within the thickness of duct's wall. (C) High magnification of duct's wall, note the presence of granules in formation and mitochondria (arrows), asterisk shows duct lumen. g, Granules. (D) Within the luminal duct, intact (arrow head) and degenerated mitochondria (arrows) can be seen between the secretory granules.

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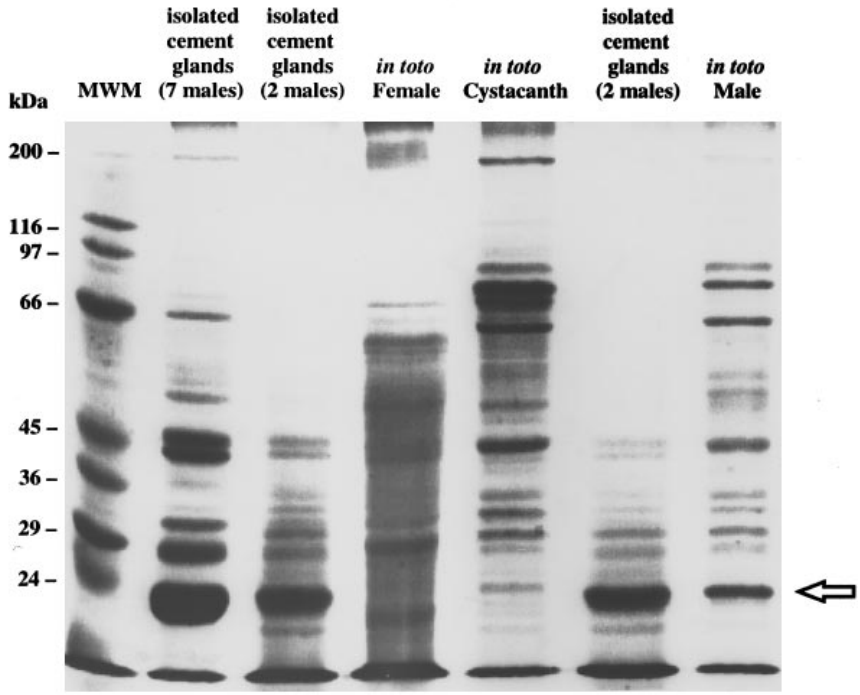

Fig. 6. Comparison of the electrophoretic protein patterns which belong to *Pomphorhynchus laevis* adult males and females, male cystacanths and isolated cement glands. MWM is the molecular weight marker. The open arrow shows the 23 kDa protein specific to cement glands.

Cleave, 1949*a*). The cytoplasmic layer contains a number of nuclei and surrounds the luminal space for cement storage (Fig. 2A), immediately adjacent to the mouth of the efferent cement duct.

Ultrastructure of the cement gland and cement duct in mature P. laevis

The glands are surrounded by a material of fibrous nature which appears as an extension of the posterior region of the ligament sac (Fig. 2B). Each gland has its own envelope which is about 0·15 μm thick (Fig. 2B). Beneath this envelope there is an outer cytoplasmic layer, with thickness about 20 μm (Fig. 2C); here the presence of a number of nuclei with diameters ranging from 10 to 22 μm can be noticed (Fig. 2D). Each nucleus has a lobed shape with an irregular outline, homogenous chromatin and zones of heterochromatin (Fig. 2D). Also in these glands there is no sign of the plasmalemma between the nuclei of the cement gland. Within the cytoplasmic layer, mainly in its peripheral portion, the occurrence of translucid vesicles was common (Fig. 2D). Moreover, this layer contains many mitochondria with lamellar cristae (Fig. 3A). Frequently, a cluster of mitochondria was noticed very close to the nucleus (Fig. 3A). Furthermore, the cytoplasm is filled with prominent rough endoplasmic reticulum (RER) (Fig. 3B) which is in close relationship with secretory granules (Fig. 3B). In contrast the Golgi apparatus was rarely observed. Within the gland lumen, the nuclei appeared to be degenerated; here, they were surrounded by a residue of cytoplasm and many electron-dense granules, namely secretory granules (Fig. 3C), measuring at least 1 μm in diameter. The granules progressively occupied the gland lumen and the cytoplasmic layer became thinner. Usually, inside the lumen, residues of cytoplasm, degenerated mitochondria and rarely intact ones were found among the secretory granules (Figs 3D and 4A, B). Electron microscopy of mature *P. laevis* cement glands permitted us to speculate on the possible mode of formation of secretory granules. Initially, from the outermost part of the cytoplasmic layer (Fig. 2C), in close contact with the RER, the electron-opaque inclusions start to be formed and surrounded by a single membrane. Between the granule membrane and its electron-dense content there is an electron-lucid space (Fig. 4C); gradually, this space is filled with a fine, granular substance (Figs 4D and 5A). Finally, in 'mature' secretory granules, the granule membrane surrounds the dense material very closely. Thus, within the 'mature' granules 2 regions are recognizable; a narrow outer granular area encloses a wide, dense, amorphous component (Fig. 5A). In many instances, the granules in formation are in close contact with fully 'mature' cement granules (Figs 4C and 5A).

A cement duct arises from each gland and appears to lead into the genital sheath, but it is not clear into which structure the ducts eventually open. The thin wall of the duct is an average of 1·6 μm in thickness (Fig. 5B). Gland duct preserved the same structural

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features of the gland's cytoplasmic layer, although the nuclei were not seen. Secretory granules and mitochondria were observed in the duct wall (Fig. 5C) and in the duct lumen (Fig. 5D).

Electrophoretic analysis of cement glands

Electrophoresis was used to separate proteins present in mature adult males, mature females and male cystacanths, all *in toto*, as well as in cement glands of 2 and 7 adult males (Fig. 6), according to standard methods. The occurrence of a protein with molecular weight of 23 kDa (arrow) can be observed in lanes belonging to adult males and male cystacanths *in toto*, and to cement glands from 2 and 7 males. The presence of this protein in glands of mature males was more obvious with an increase in number of cement glands, whereas this protein was not found in female specimens of acanthocephalan.

DISCUSSION

Meyer (1933, on page 459 of his monograph) suggested that the secretion is formed by the holocrine mode of transformation of plasma. Exocrine glands may be classified as 1 of 3 physiological types: merocrine, apocrine and holocrine types (Patt & Patt, 1969). Our results revealed that in *P. laevis* the cement glands are holocrine. This conclusion is based on the following points. (i) The occurrence of nuclei in degeneration as well as that of mitochondria and residues of cell cytoplasm among the secretory granules within the gland lumen. (ii) In merocrine and apocrine glands, the Golgi complex is abundant and participates actively in secretion, nonetheless, Golgi apparatus within the cytoplasmic layer of gland of *P. laevis* was rarely encountered.

According to Van Cleave (1949*a*), the cement gland produces secretions which function to cement the bodies of the 2 partners together during copulation. Nicholas (1973) reported that during copulation, the everted male bursa is sealed around the female gonopore by the secretion of the cement glands. Concerning the bursa and cement function, Dunagan & Miller (1973) described 2 types of gland-like cells at the margin of this extrusive organ of male *Fessisentis fessus*; these cells are situated in an appropriate position for the release of a 'catalyst', apparently involved in copulation cap formation. Dunagan & Miller's (1973) interpretation was based on no cytochemical or ultrastructural information. Indeed, in reference to the gland-like cells, the authors wrote, 'we are unable to establish an opening for these structures'. Regarding the presence of gland-like cells in *P. laevis* bursa, careful study of a large number of sections revealed the occurrence of only 2–3 nuclei, immersed in syncytial tissue of the tegument along the peripheral margin of the bursa (B. S. Dezfuli, unpublished). There is no sign of 2 types of cells, or of their presence in high numbers (30), as reported by Dunagan & Miller (1973) for *F. fessus*.

In male acanthocephalans, the cement glands produce a mucilaginous (Van Cleave, 1949*a*), proteinaceous material (Haley & Bullock, 1952) called cement. Soon after insemination is over, the cement becomes hardened to form a 'copulatory cap' around the genital extremities of the female worm (Van Cleave, 1949*a*). The occurrence of this cap over the posterior end of some male acanthocephalans was reported by Abele & Gilchrist (1977) and Moore & Bell (1983). Abele & Gilchrist (1977) suggested that 'homosexual rape may be due to poor sex recognition'. Moreover, they concluded that acanthocephalans conform to a parental investment model and the evolution of the cement gland and sexual behaviour is a result of sexual selection.

With regard to capping behaviour among acanthocephalans, Abele & Gilchrist (1977) formulated the following 2 hypotheses: (i) the cap prevents the escape of sperm from the female body; (ii) the cap formation evolved in response to sexual selection and functions in preventing subsequent inseminations. Regarding the last hypothesis, Crompton (1985) emphasized that the cap attached to the gonopore of a female could have many advantages for the genes of the male which inseminated the partner, then secreted the cement.

Regarding the nuclei of cement glands, Kaiser (1893) considered them to be 'Drüsensyncytium' (gland-syncytium). Meyer (1933) offered no objection to the use of the term 'syncytium' for Eoacanthocephala, but rejected this interpretation for Palaeacanthocephala and with regard to this class suggested that the term 'gland-syncytium' should not be used since, in all cases where several 'nuclei' occur, they are nuclear fragments. This definition was accepted and elaborated by Van Cleave (1949*a*) as follows, '…fragmentation of the giant nuclei of the glands characteristic of the Archiacanthocephala results in the production of a large number of nuclear fragments which become dispersed throughout the cortical region of each gland in the Palaeacanthocephala'. Because of the difficulties inherent to obtaining the earliest larval stages of *P. laevis*, the observations presented here were made on the structure of cement glands from cystacanth and adult acanthocephalan. Thus, our knowledge is too limited to make any generalizations regarding Meyer's (1933) and Van Cleave's (1949*a*) interpretation for Palaeacanthocephala. In light of our current understanding of cement glands of *P. laevis*, it is evident that a great deal remains to be discovered about the cement apparatus in the Acanthocephala.

This report provides data regarding the secretory function of the cement gland duct. Based on the

similarity of the structural features between the duct and the gland's cytoplasmic layer and the lack of nuclei within the thickness of the wall of the former, it could be concluded that the duct is a cytoplasmic extension of the gland itself, thus a secretory function could be attributed to it. This function relies also on the occurrence of some secretory granules within the thickness of the duct wall. Nevertheless, based on the low granule numbers here, it seems probable that the duct has a minor role in producing cement.

The present investigation was undertaken to gain information on the ultrastructure and probable occurrence of cement in glands of extraintestinal *P. laevis* recovered from its paratenic host (*A. a. alborella*). As mentioned in the previous section, the gland of immature males had a compact structure with no lumen. Concerning the cement, in none of extraintestinal males was it encountered. Accordingly, the glands in this group of males do not produce cement. Apparently, the secretion of cement as well as the maturity of acanthocephalan gonads occur within the digestive tract of the definitive host (e.g. chub).

With reference to the nature of secretion of cement glands, histological staining was used to gain data on the biochemical nature of the secretion. According to Parshad & Crompton (1981), Heidenhain's iron haematoxylin stained the cement a blue-black colour, thus the cement is likely to contain protein. The luminal area of cement glands of *P. laevis* with the above-mentioned staining appeared blue-black. Furthermore, the PAS stain used here was necessary due to the range of substances whose presence could be revealed with this stain (Pearse, 1968). *P. laevis* cement glands showed a negative reaction to PAS, thus substances such as polysaccharides, glucosaminoglycans and glucosaminoglucuronoglycans, glycoproteins and glycopeptide, glycolipids, unsaturated lipids and phospholipids were absent from cement glands. The PAS staining supported the suggestion that the cement glands secrete a proteinaceous material.

Based on the histological data that suggested that the secretory product is proteinaceous, electrophoretic techniques were used to gain further information about the cement. The comparison of electrophoretic protein patterns belonging to different sexes of *P. laevis* as well as in cystacanths and isolated cement glands showed that a protein of about 23 kDa is absent only from female individuals. Interestingly, in isolated adult cement glands this protein appeared to be the major component of the gland proteins, and its concentration increased with an increasing number of cement glands. It is noteworthy that in male cystacanths the above-mentioned protein was present in low concentrations, probably because in this stage the cement glands had not reached their definitive dimension and functionality.

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