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Changes in Esophageal Gland Activity During the Life Cycle of *Nacobbus aberrans* (Nemata: Pratylenchidae)[1]

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Abstract: Electron and light microscopy were used to study the dorsal gland (DG) and the two subventral glands (SvG) of seven developmental phases of *Nacobbus aberrans:* pre-parasitic second-stage juveniles (J2), parasitic J2, third- (J3) and fourth- (J4) stages, migratory females, young sedentary females, and mature sedentary females. In each developmental phase the level of esophageal gland activity was estimated by the abundance of organelles associated with secretory pathways, including endoplasmic reticulum, ribosomes, Golgi, multivesicular bodies, and secretory granules. All esophageal glands were metabolically active in all J2 examined, although only in parasitic J2 were there numerous secretory granules in the esophageal gland extensions and ampullae. No evidence of secretory activity was observed in the esophageal glands of the coiled and relatively inactive J3 and J4, nor in migratory females; these stages apparently do not feed. Observations suggest that reserves stored by J2 sustain three ecdyses and the migratory female's search for a feeding site and induction of a syncytium. Feeding activity is resumed in young and mature sedentary females, in which the DG is highly active and enlarged. The SvG are metabolically active, but with little synthesis of secretory granules, suggesting that in sedentary females the SvG may have physiological roles other than digestion.

Key words: esophageal glands, feeding biology, gland activity, *Nacobbus aberrans,* nematode, Pratylenchidae, ultrastructure.

Investigations of the dorsal gland (DG) and the two subventral glands (SvG) of parasitic Tylenchida have begun to unveil the complex role of these esophageal glands in the life cycle and in parasitism (Baum et al., 1996; De Boer et al., 1996; Endo, 1993; Hussey and Mims, 1990; Ray et al., 1994; Willats et al., 1995; Wyss, 1992; Zunke, 1990). The SvG presumably play a role in hatching of *Meloidogyne javanica* (Treub) Chitwood (Bird, 1968). Video-recording studies of infective second-stage juveniles (J2) of *Heterodera schachtii* Schmidt and *M. incognita* (Kofoid & White) Chitwood suggest that esophageal gland secretions are not used for root penetration (Wyss and Zunke, 1986; Wyss et al., 1992). In *M. incognita* all three esophageal glands seem to participate in the intercellular migration of parasitic J2 through roots; conversely, in *H. schachtii* and *Pratylenchus penetrans* (Cobb) Filipjev & Schuurmanns Stekhoven, none of the esophageal glands seem to be involved in intracellular migration (Wyss, 1992; Wyss et al., 1992; Zunke, 1990). In all species examined it appears that DG secretions play a role in induction and maintenance of sedentary feeding sites. SvG secretions, once thought to flow exclusively into the intestine, recently were detected as a component of stylet secretions (Davis et al., 1994; Endo, 1987, 1993; Goverse et al., 1994).

To perform such a range of functions, the esophageal glands must have a precisely timed developmental program, in which perception of environmental conditions and signals, neural coordination, genetic expression and suppression, and glandular secretory pathways are coordinated throughout the life cycle of the nematode. Currently, our limited understanding of developmental biology of esophageal glands is based primarily on *Meloidogyne* and *Heterodera,* both sedentary endoparasites. In these two heteroderids, the SvG seem to be most active in the infective J2, with a reduction of secretory activity coordinated with the onset of parasitism. Conversely, days after the initial induction of a feeding site the DG activity reaches a peak and remains high throughout the life cycle (Endo, 1984, 1987, 1993; Endo and Wergin, 1988; Hussey and Mims,

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1990). In all other parasitic Tylenchida, representing many parasitic habits, the developmental biology of esophageal glands remains virtually uninvestigated, except for two electron microscopy studies that suggest that all three esophageal glands remain active throughout the life cycles of the migratory endoparasites *P. penetrans* and *Ditylenchus dipsaci* (Kuhn) Filipjev (Endo et al., 1997; Shepherd and Clark, 1983).

To expand the understanding of the role of esophageal glands in parasitism in a broader range of taxa and parasitic habits, we selected *Nacobbus aberrans* (Thorne) Thorne & Allen (Pratylenchidae), an economically important parasite of sugarbeet and vegetables in the United States and in some Latin American countries (Inserra et al., 1985). In its life cycle of 36 to 38 days at 25°C, the migratory J2 molts into a relatively inactive, coiled, third- (J3) and fourth- (J4) stage juvenile. The vermiform, immature females are migratory endoparasites that, in response to an unknown signal, become sessile, swell for production of eggs, and induce the formation of a syncytium that exhibits only minor differences from the syncytia of *Heterodera* (Inserra et al., 1985).

Since the life cycle of *N. aberrans* includes a migratory phase similar to pratylenchids and a sedentary phase to heteroderids, we suggest that this species is a useful model for understanding the role of esophageal glands in both feeding biologies. In this developmental study of esophageal glands we combined electron and light microscopy to estimate the esophageal gland activity throughout seven developmental phases in five life stages of *N. aberrans*. We also briefly examined the esophageal gland activity of *Pratylenchus vulnus* Allen & Jensen to allow comparison with an exclusively migratory parasitic habit.

Materials and Methods

A single Mexican isolate of *N. aberrans* maintained on tomato (*Lycopersicon esculentum* Mill) in the University of California-Riverside (UCR) Quarantine Facility was the source of specimens at seven developmental phases examined in this study: active preparasitic J2; active parasitic J2, J3, J4; migratory females; young sedentary females; and mature sedentary females. Active preparasitic J2 were obtained by hatching eggs in water, and subsequently they were kept for 48 hours at room temperature for full extension of the esophageal glands. Active parasitic J2 and active migratory females were obtained by gentle blending of infected roots, followed by sieving and selection of specimens under a dissecting microscope. The J3, J4, young sedentary females with up to 10 laid eggs, and mature sedentary females with large egg masses were removed from infected roots under a dissecting microscope. The vitality of sedentary females was inferred by absence of fungi colonizing their bodies and by the presence of visiting active males. The specimens were transferred to 1% sodium chloride solution and immediately prepared for microscopy.

For light microscopy, the specimens were mounted on temporary slides with the anesthetic 0.1 M sodium azide, modified from Sulston and Hodgkin (1988), and observed with an interference-contrast microscope (IM). At least 15 specimens of each developmental phase were observed with IM. For comparison, the same procedure was used to observe different life stages of *P. vulnus*.

For transmission electron microscopy, all developmental phases were fixed at room temperature with 2% osmium tetroxide solution in 0.1 M sodium cacodylate buffer at pH 7.2, with 1.5 mM of calcium chloride added. Depending on the life stage, 20 to 40 minutes later the specimens were cut in two and returned to the fixative for a total fixation time of 2.5 hours. Following a thorough rinse with 1% sodium chloride, the specimens were stained with aqueous 1% uranyl acetate at 4°C for 15 hours, and rinsed again. Specimens were placed in 5% agar blocks, dehydrated with serial acetone solutions, and infiltrated and embedded in Spurr resin (Spurr, 1969).

An MT 6000 Sorval ultramicrotome and diamond knife were used to obtain serial sil-

ver-to-gold sections, which were post-stained with lead citrate (Reynolds, 1963). A Hitachi H600 transmission electron microscope (TEM) was operated at 75 kV. Four to eight specimens in each developmental phase were examined with TEM, and at least two specimens in each phase were examined throughout the entire length of the esophagus.

Results

Nacobbus aberrans

Pre-parasitic J2: The DG and SvG ampullae and extensions of pre-parasitic J2 were slender and appeared to lack contents (Fig. 1A). The larger, basal portions of the esophageal glands, herein referred to as lobes, were well extended, and each lobe had a distinct

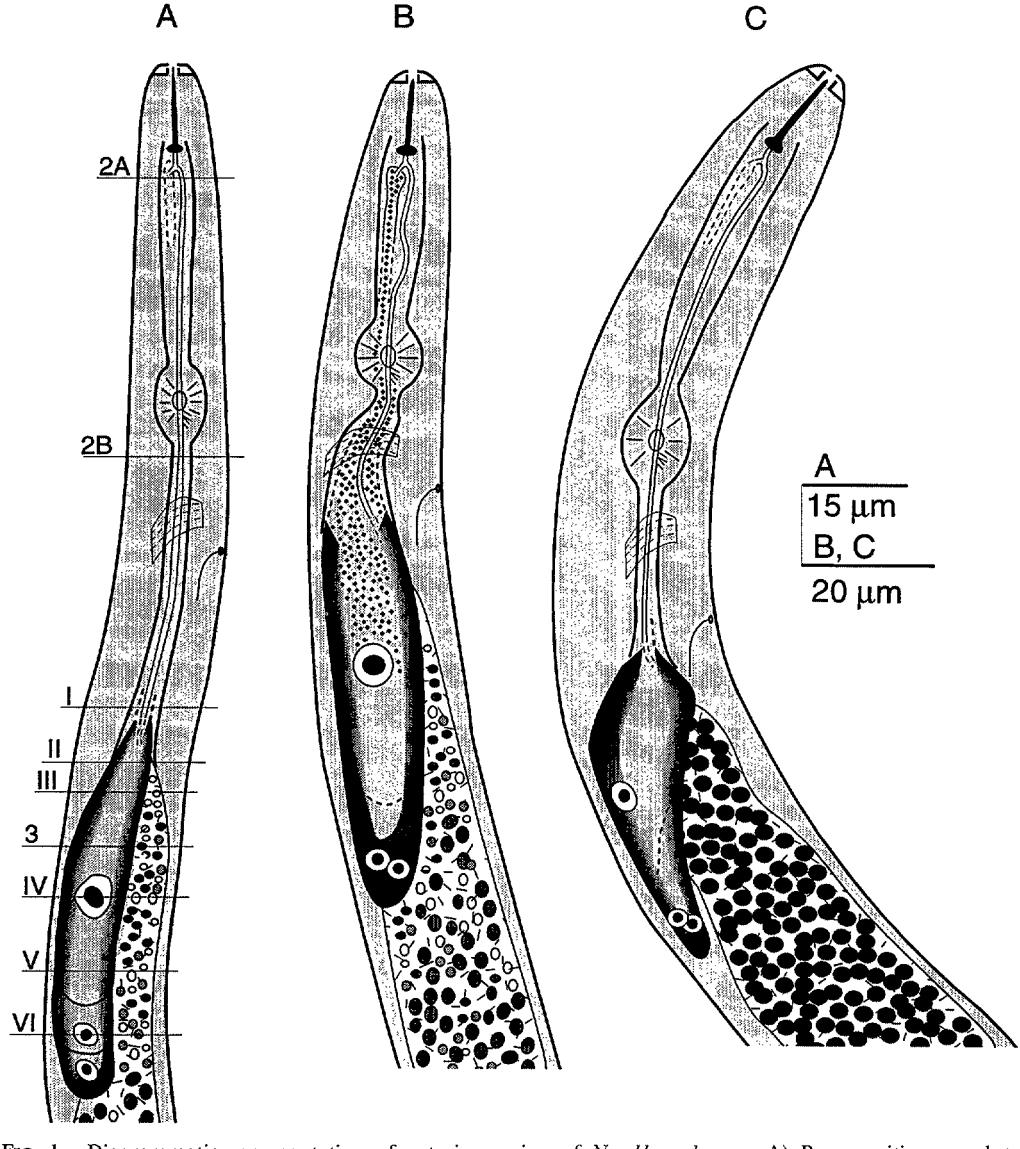

FIG. 1. Diagrammatic representation of anterior region of *Nacobbus aberrans*. A) Pre-parasitic second-stage juvenile (J2). Arabic numbers indicate the relative position of electron micrographs of transverse sections in the various figures. Roman numbers indicate the relative position of transverse sections shown in Fig. 4A. B) Parasitic J2. C) Third- or fourth-stage juvenile.

nucleus. In TEM the DG ampulla and extension had only irregularly-shaped, electron-lucent secretory granules, which were interpreted as depleted secretory granules (Fig. 2A). The cytoplasm of the DG lobe had abundant endoplasmic reticulum, ribosomes, mitochondria, Golgi bodies, multivesicular bodies, and secretory granules (Fig. 3A). Secretory granules with electron-lucent contents undergoing sorting and condensa-

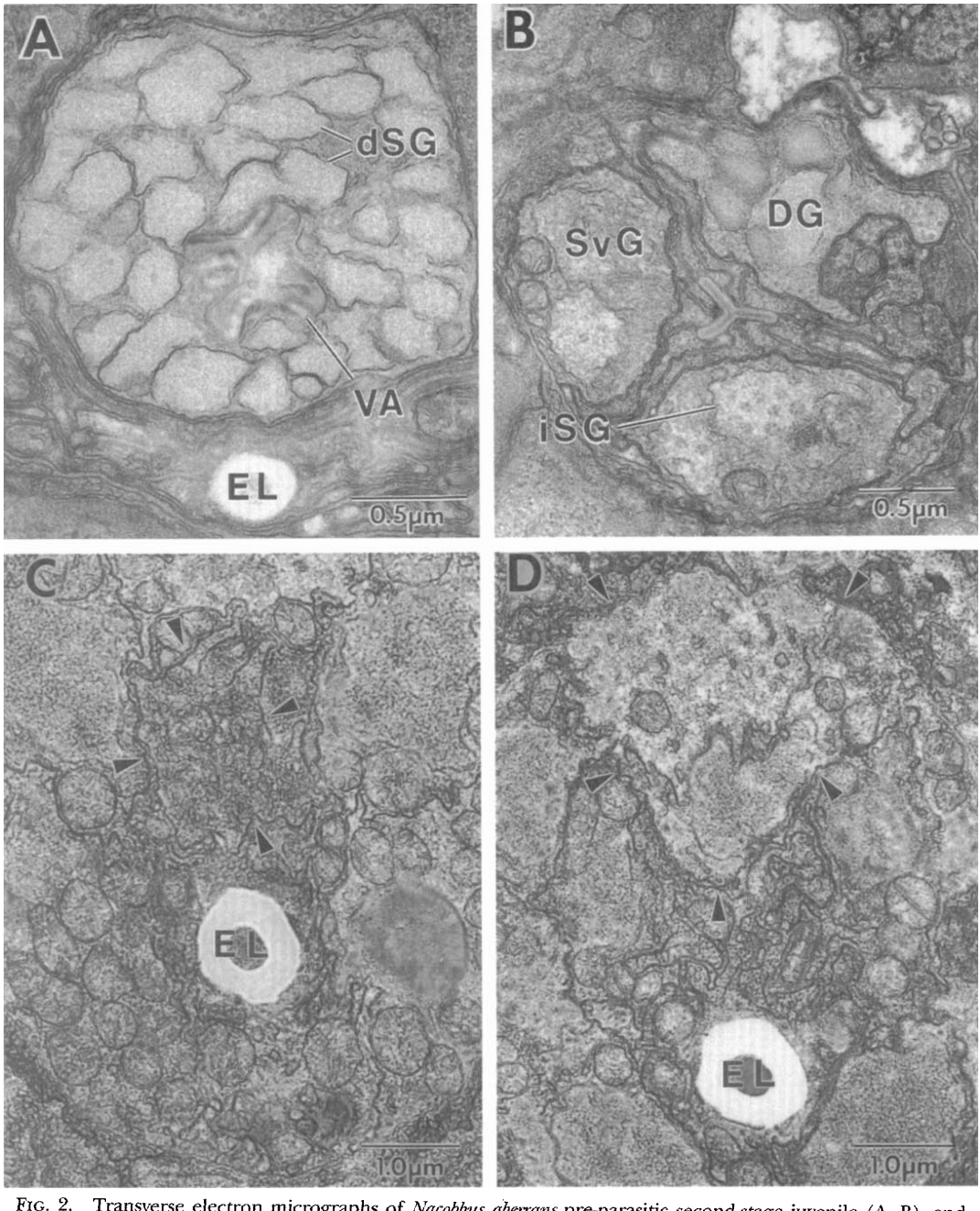

FIG. 2. Transverse electron micrographs of *Nacobbus aberrans* pre-parasitic second-stage juvenile (A, B), and migratory female (C, D). A) Dorsal gland (DG) ampulla with depleted secretory granules (dSG) surrounding gland valve apparatus (VA). EL: Esophageal lumen. B) DG and subventral gland (SvG) extensions with immature secretory granules (iSG). C) DG ampulla, surrounded by arrowheads. D) DG extension, surrounded by arrowheads.

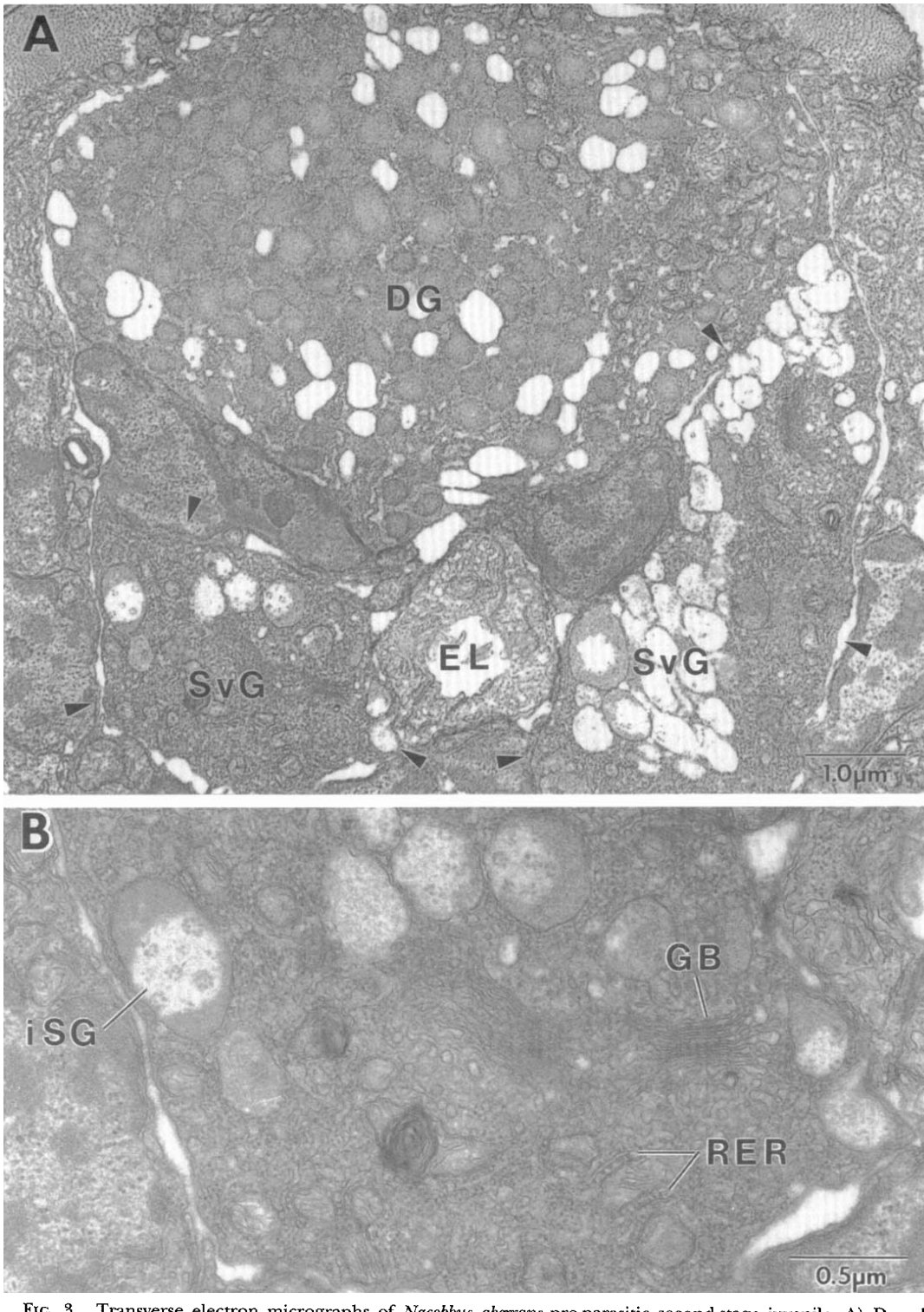

FIG. 3. Transverse electron micrographs of *Nacobbus aberrans* pre-parasitic second-stage juvenile. A) Dorsal gland (DG) and subventral glands (SvG), surrounded by arrowheads. EL: Esophageal lumen. B) Enlargement from 3A showing immature secretory granules (iSG), Golgi bodies (GB), and rough endoplasmic reticulum (RER).

tion were interpreted as immature secretory granules, and were present throughout the DG lobe. Secretory granules with uniformly electron-dense contents were interpreted as mature granules, and were clustered in the upper region of the DG lobe, above the nucleus. The SvG extensions and lobes had immature secretory granules only (Figs. 2B, 3). The same organelles described above for the DG lobe were found in the SvG lobes, with the exception of multivesicular bodies.

In serial, transverse TEM the esophageal glands of pre-parasitic J2 were reduced to narrow cell processes from their anterior end to about the level of the esophagus-intestine junction (Fig. 4A). More posteriorly, the three esophageal gland lobes alternately widened and narrowed along their length. This arrangement was consistent in all specimens examined, with minor differences in the diameter of each esophageal gland.

Parasitic J2: In parasitic J2, the DG ampulla and extension were dilated and

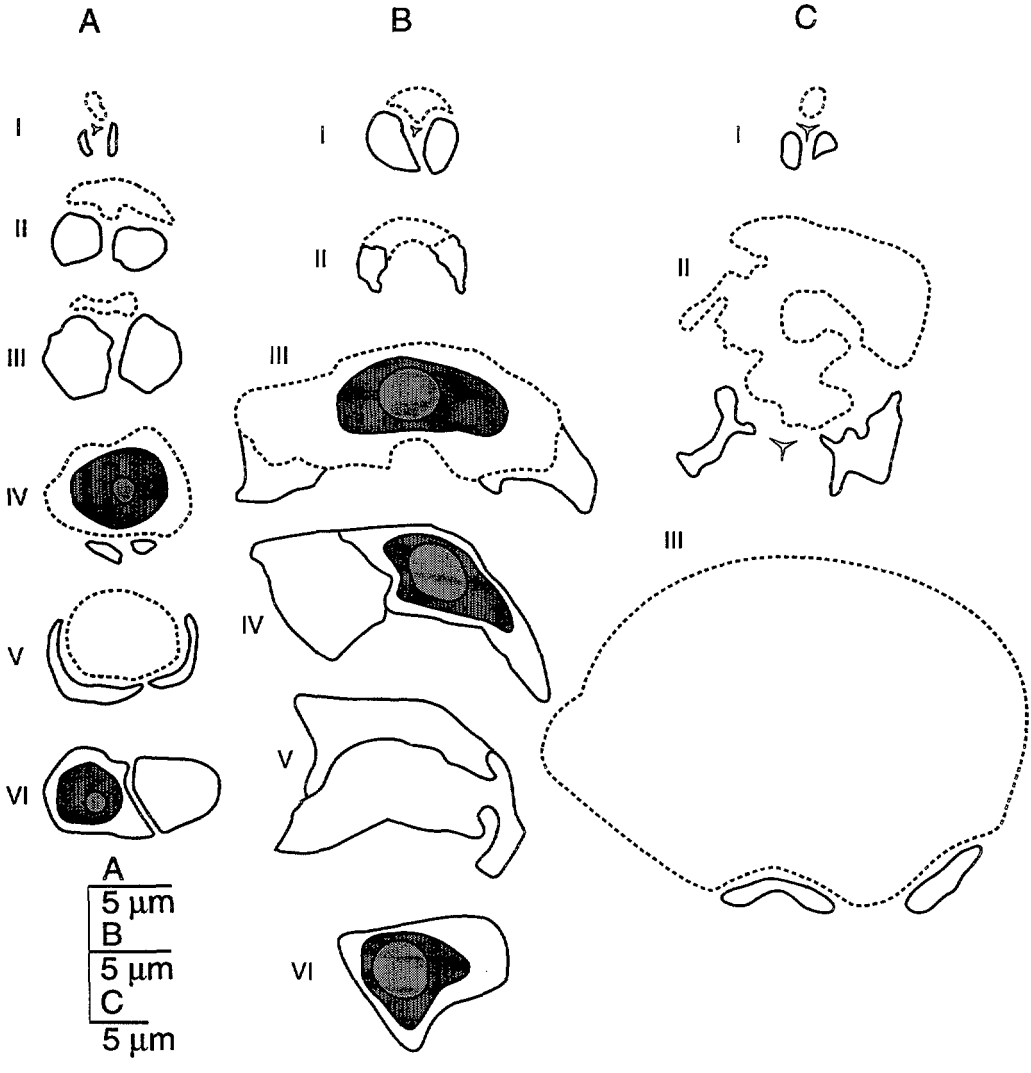

FIG. 4. Diagrammatic representation of electron micrographs of transverse sections showing the relative arrangement of esophageal glands in *Nacobbus aberrans*. Roman numbers indicate the relative position of transverse sections in Figs. 1A and 5. Dashed lines represent dorsal gland, and continuous lines represent subventral glands. A) Pre-parasitic second-stage juvenile. B) Migratory female. C) Sedentary female.

packed with secretory granules (Fig. 1B). The SvG ampullae and extensions had many secretory granules, as did the three esophageal gland lobes throughout the anterior half of their length. In contrast to preparasitic J2, the SvG of parasitic J2 were about the same length. The nuclei and nucleoli were well developed in all three esophageal glands.

J3 and J4: These life stages were barely distinguishable from one another on the basis of body and genital primordium lengths. In water, these stages were largely inactive, and only rarely did they uncoil from their typical spiral shape. No activity or contents were visible with IM throughout the entire length of the esophageal glands (Fig. 1C). The cell membranes that demarcate each of the gland lobes could not be discerned, but the glands were smaller than in parasitic J2. The three esophageal gland nuclei were reduced when compared to parasitic J2 but had the same relative positions in the glands.

Migratory female: In migratory females, the outlines and the contents of the DG and SvG ampullae and extensions could not be discerned with IM, and no signs of secretory activity or secretory granules were observed in any of the three esophageal gland lobes (Fig. 5A). No secretory granules were observed with TEM in the narrow DG ampulla and extension (Fig. 2C, D), nor in the SvG ampullae and extensions. The DG and SvG lobes lacked secretory granules but had many mitochondria, multivesicular bodies, and small vesicle-like structures (Fig. 6A, B).

The esophageal glands in migratory females were reduced to narrow cell processes throughout the long isthmus, but posterior to the esophagus-intestine junction the DG became predominant (Fig. 4B). Posteriorly, the SvG were adjacent and arranged either laterally or dorso-ventrally.

Young sedentary female: The esophageal glands of sedentary females, either young or mature, were remarkably different from those of migratory females (Fig. 5B). The DG of sedentary females had an enormous ampulla and extension packed with secretory granules, which were seen throughout the metacorpus and isthmus. The DG lobe broadened in the isthmus region to occupy about half of the diameter of the female's "neck," but the actual length and width of the DG lobe was variable among specimens. SvG ampullae, extensions, and lobes could not be seen with IM but were reconstructed from serial electron micrographs. The DG ampulla of young sedentary females was packed with mature and depleted secretory granules (Figs. 7A,B). In some specimens the contents of the DG ampulla were polarized, with secretory granules clustered around the gland valves and abundant endoplasmic reticulum and multivesicular bodies restricted to the basal region of the ampulla. Throughout the procorpus and isthmus, the cytoplasm of the DG extension had mature secretory granules, endoplasmic reticulum, multivesicular bodies, ribosomes, and mitochondria (Fig. 7C). The cytoplasm of the DG lobe had abundant secretory granules (Fig. 8A) and was always organized as follows: mitochondria were concentrated at the edges of the cell; clusters of mature secretory granules, as well as some degenerating granules and multivesicular bodies were localized in the upper part of the DG lobe; and abundant endoplasmic reticulum, ribosomes, and small electron-lucent, membrane-bound vesicles were dispersed throughout the gland lobe. The cytoplasm of SvG had a complete set of organelles, although the secretory granules always seemed deteriorated throughout the SvG extensions and lobes. The contents of the secretory granules were electron-lucent and dispersed, their membranes were ruptured, and they often seemed to fuse with large multivesicular bodies (Fig. 8B,C).

Mature sedentary female: Organelles in the DG ampulla, extension, and lobe of mature sedentary females were abundant as in young sedentary females, and they included mature secretory granules, mitochondria, endoplasmic reticulum, ribosomes, and small, membrane-bound vesicles (Fig. 9A). Similarly to young sedentary females, only depleted secretory granules were present in the SvG ampullae of mature sedentary females (Fig. 7D). The SvG extensions and lobes contained endoplasmic reticulum,

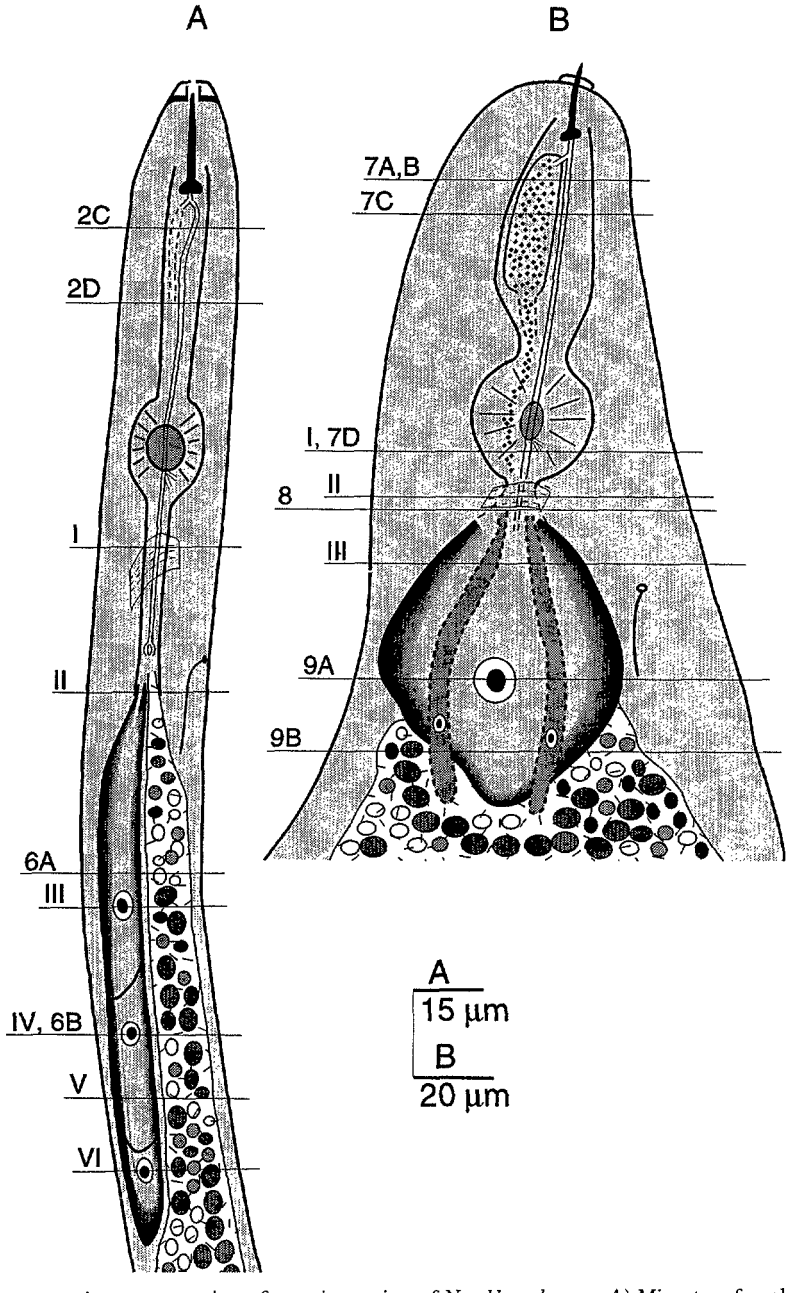

FIG. 5. Diagrammatic representation of anterior region of *Nacobbus aberrans*. A) Migratory female. B) Sedentary female. Arabic numbers indicate the relative position of electron micrographs of transverse sections in the various figures. Roman numbers indicate the relative position of transverse sections shown in Figs. 4B and 4C. Dashed outlines delimiting subventral glands are inferred from serial electron micrographs.

Golgi bodies, mitochondria, lipid droplets, and degenerated secretory granules. These granules fused with each other and with large multivesicular bodies (Fig. 9B).

In sedentary females, the three esophageal glands were about equal in diameter and irregular in shape in the isthmus; posteriorly, the DG enlarged considerably, whereas the SvG retained about the same diameter throughout the lobe (Fig. 4C).

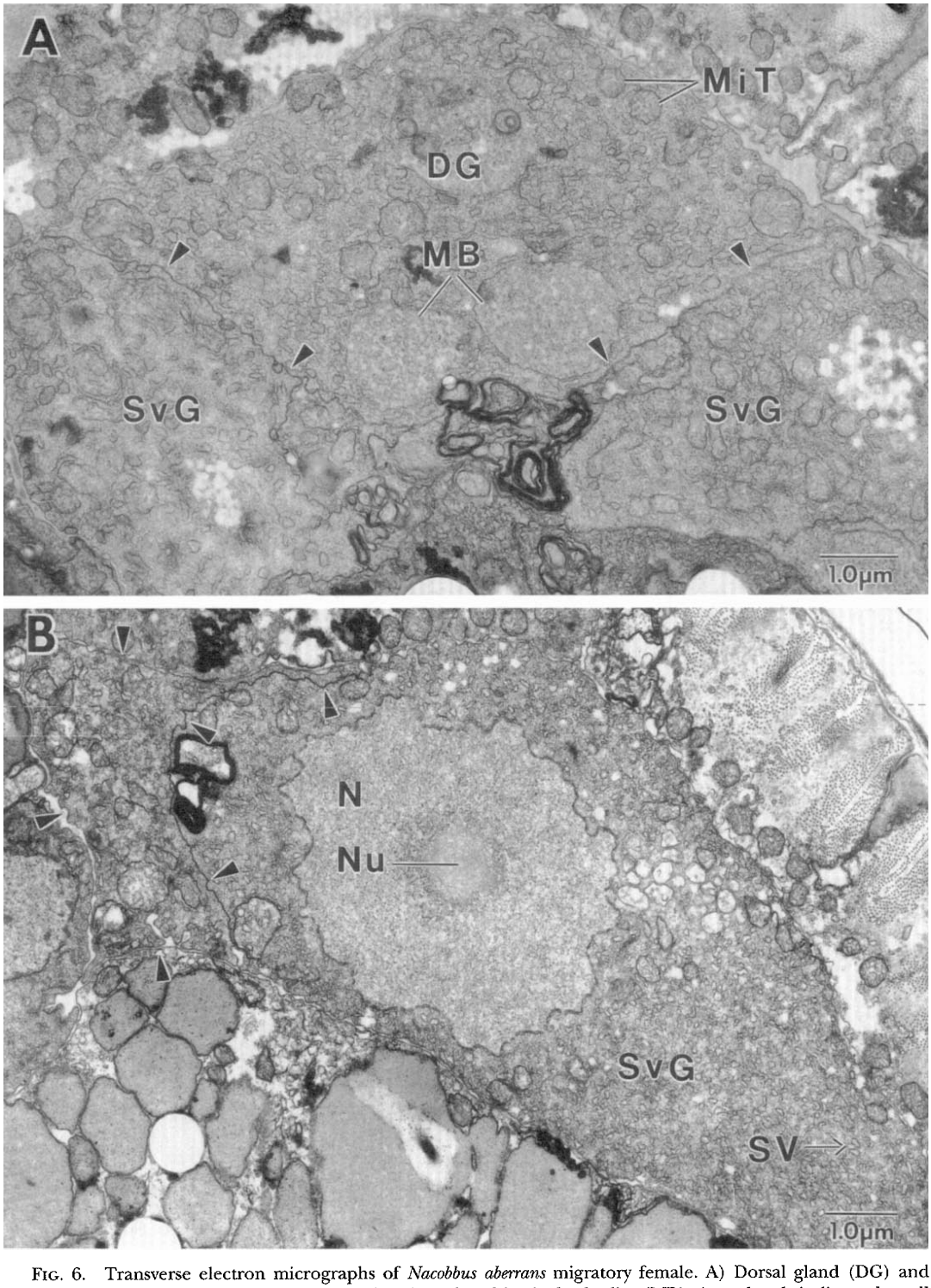

FIG. 6. Transverse electron micrographs of *Nacobbus aberrans* migratory female. A) Dorsal gland (DG) and subventral glands (SvG) with mitochondria (MiT), and multivesicular bodies (MB). Arrowheads indicate the cell membranes that separate DG and SvG. B) Two SvG with nucleus (N), nucleolus (Nu), and small vesicles (SV). Arrowheads surround the smaller SvG.

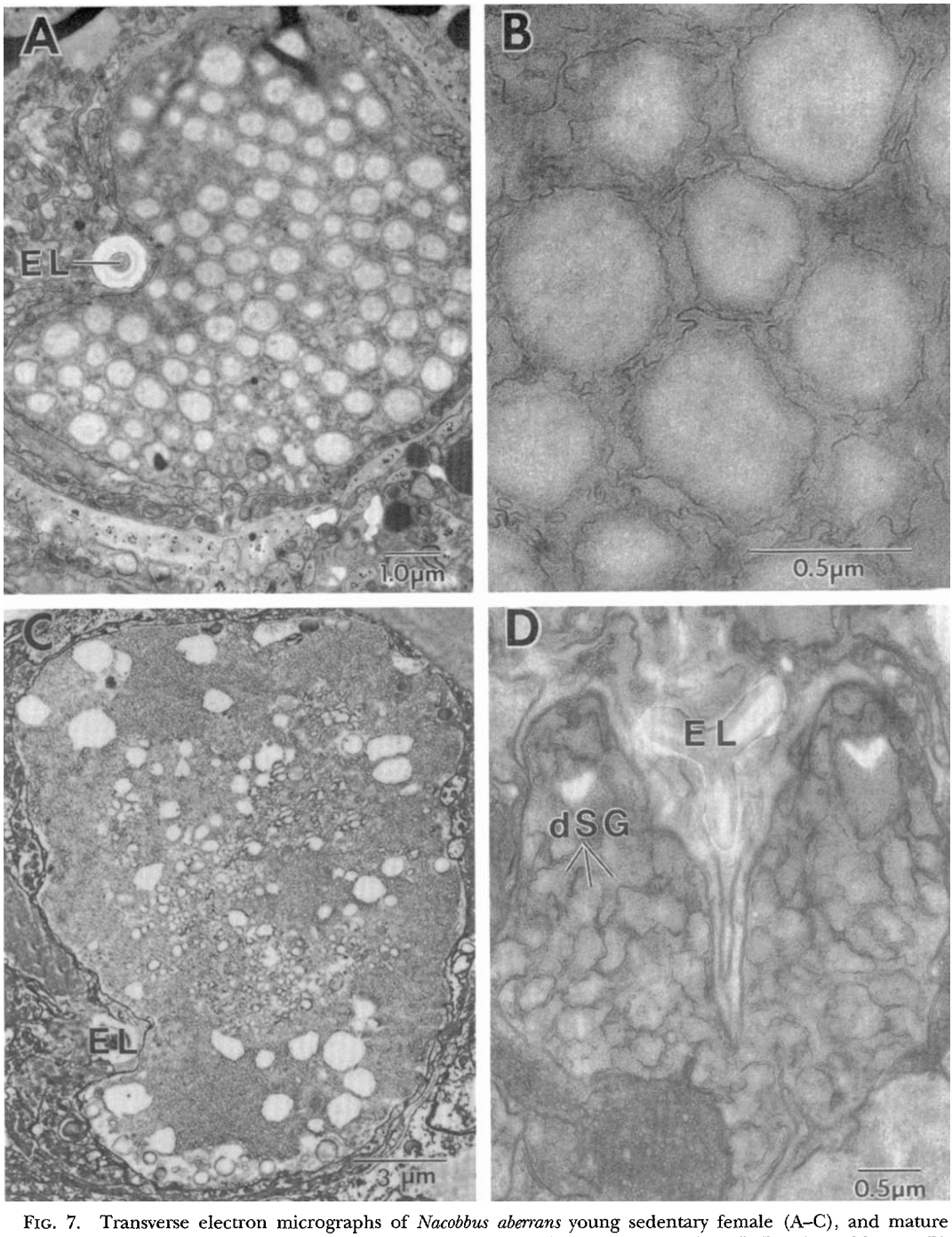

FIG. 7. Transverse electron micrographs of *Nacobbus aberrans* young sedentary female (A–C), and mature sedentary female (D). A) Dorsal gland (DG) ampulla packed with secretory granules. EL: Esophageal lumen. B) Enlargement from 7A showing secretory granules. C) DG extension packed with organelles. D) Subventral gland ampullae with depleted secretory granules. (dSG).

Pratylenchus vulnus

In all *P. vulnus* life stages examined with IM, the DG and SvG ampullae and extensions were swollen with secretory granules, although they seemed relatively less abundant in the J2. The three esophageal gland lobes were expanded, with many secretory

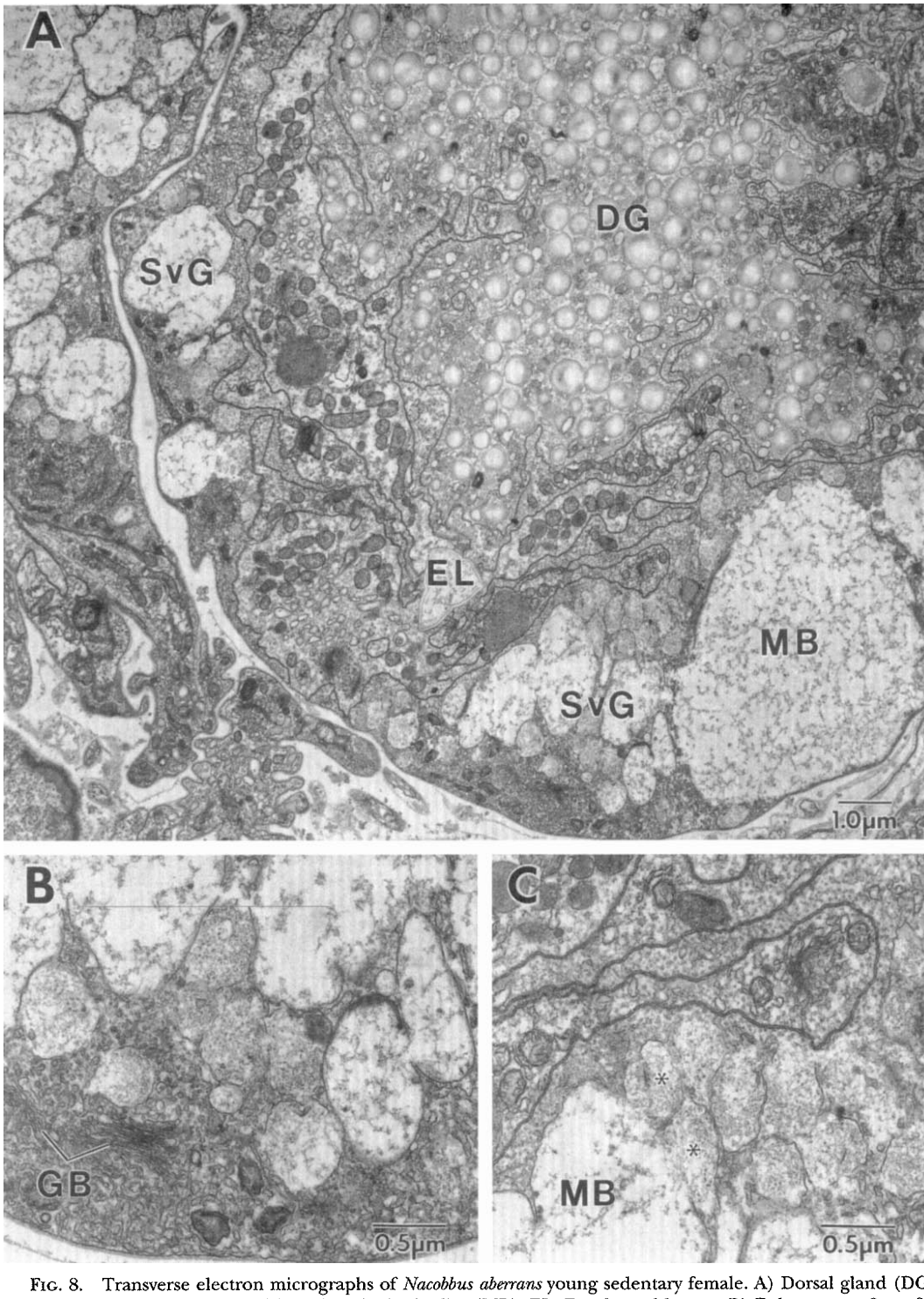

FIG. 8. Transverse electron micrographs of *Nacobbus aberrans* young sedentary female. A) Dorsal gland (DG), and subventral glands (SvG) with multivesicular bodies (MB). EL: Esophageal lumen. B) Enlargement from 8A. GB: Golgi bodies. C) Enlargement from 8A. Asterisks indicate degenerated secretory granules being incorporated into multivesicular bodies.

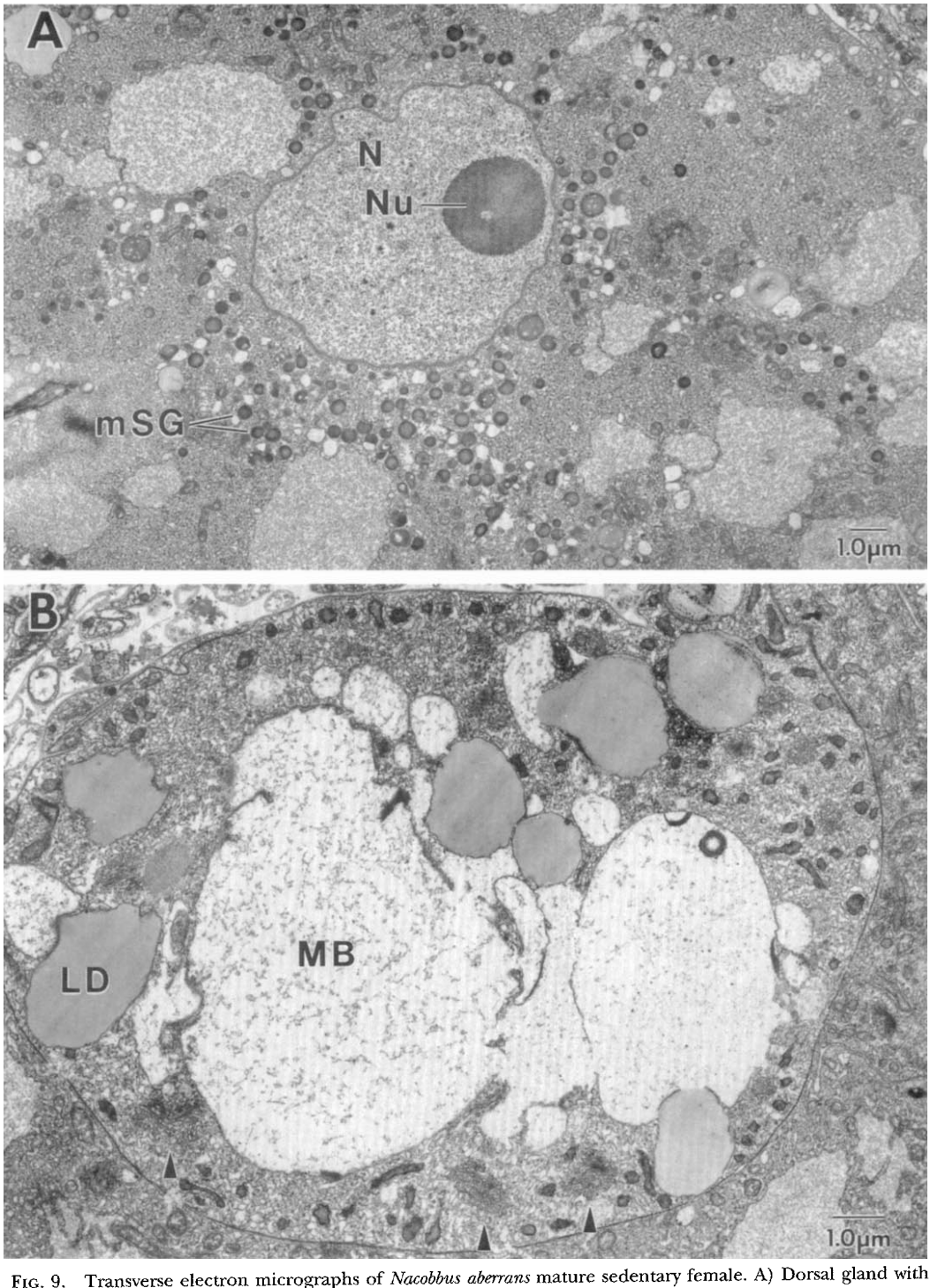

FIG. 9. Transverse electron micrographs of *Nacobbus aberrans* mature sedentary female. A) Dorsal gland with numerous mature secretory granules (mSG), nucleus (N), and nucleolus (Nu). B) Subventral gland with large multivesicular bodies (MB), lipid droplets (LD), and Golgi bodies (arrowheads).

granules concentrated in the upper part of the gland lobes, and with a distinct nucleus and nucleolus in each gland. In comparison to other life stages, J2 had the shortest and narrowest esophageal gland lobes, and their nuclei and nucleoli were notably smaller.

Discussion

This work helps to clarify the complex developmental biology of esophageal glands in a parasitic Tylenchida. Our findings suggest that, in *N. aberrans*, esophageal gland activity changes markedly throughout pre-parasitic and parasitic, migratory, and sedentary life stages. Esophageal gland activity is inferred by the abundance of organelles associated with secretory pathways including endoplasmic reticulum, ribosomes, Golgi, multivesicular bodies, and secretory granules (Kazumasa, 1989; Lodish et al., 1995).

Although the DG and SvG of *N. aberrans* pre-parasitic J2 are active, as suggested by the abundance of organelles in the gland lobes, the DG and SvG extensions and ampullae were not swollen with continuously migrating secretory granules, as they were in parasitic J2. We hypothesize that a secretory peak, with accumulation of secretory granules in the esophageal gland extensions and ampullae, is triggered by root exudates or actual root invasion. The presence of some depleted secretory granules in the DG ampulla of pre-parasitic J2 is interpreted as an artifact of the incubation of pre-parasitic J2 in water.

In *N. aberrans* parasitic J2, all three esophageal glands seem metabolically active, with ampullae, extensions, and lobes swollen with secretory granules. A role in migration might be questioned, however, because *N. aberrans* J2 apparently migrate intracellularly, making their way with vigorous stylet thrusts (Clark, 1967). Video-recording studies demonstrated intracellular migration for *P. penetrans* and *H. schachtii* J2 (Wyss, 1992; Zunke, 1990). In *N. aberrans* parasitic J2, a feeding role for the active esophageal glands is plausible since reports suggest that this life stage does feed, inducing subtle cellular and histological changes in addition to cortical lesions. Host changes associated with parasitic J2 include slight root swelling, granulation of cytoplasm, and hypertrophy of nuclei (Castillo and Marban, 1982; Inserra et al., 1983, 1985). Our observations suggest that, in *N. aberrans* parasitic J2, all three esophageal glands are active and may be involved in feeding, as probably is also the case for *P. penetrans* juvenile stages recently studied with TEM (Endo et al., 1997) and for *P. vulnus* examined with IM.

Contrary to the case in parasitic J2, there is no evidence of esophageal gland activity in *N. aberrans* J3 and J4, with DG and SvG ampullae, extensions, and lobes lacking secretory granules. The J3 and J4 are the least understood of all *N. aberrans* life stages. These coiled stages were reported as relatively inactive and living mainly within the roots for 12 days at 25°C before molting into migratory females (Clark, 1967; Inserra et al., 1983) but, under potato field conditions, J4 persisted in a quiescent state for up to 25 days (Gonzáles et al., 1989, cited in Canto, 1992). The J3 and J4 have been associated with root necrosis and swelling, cytoplasmic changes, cell and nucleus hypertrophy, and initiation of syncytia (Canto, 1992). Our observations of apparently inactive esophageal glands in J3 and J4 suggest that these are non-feeding stages. We further suggest that J3 and J4 are facultatively quiescent in conditions unsuitable for development and feeding, such as overwintering. Notably, only J3 and J4 are found in stored potato tubers (Costilla, 1985, cited in Canto, 1992). These stages accumulate exceptionally large amounts of lipid droplets in their intestine, coloring the body dark brown and obscuring morphological features, including the genital primordium. Facultative quiescence of J3 and J4 might explain the variation in reports of the duration of the *N. aberrans* life cycle.

No evidence of major secretory activity was observed with TEM in any of the three esophageal glands of *N. aberrans* migratory females. These migratory females are thought to leave and re-enter roots over a

span of about 7 days, searching for a favorable site to induce syncytia, away from root lesions induced by migratory J2 (Inserra et al., 1983). If migratory females were the source of inoculum, they became sessile within 2 days, and syncytia were observed within 5 days (Sialer, 1990). Based on the present study, we hypothesize that migratory females do not feed and that esophageal gland activity is not triggered until a suitable site for induction of syncytia is located. Notably, no reports suggest that migratory females feed or induce cell or tissue alterations before they become sessile.

Our hypothesis that J3, J4, and migratory females do not feed implies that parasitic J2 accumulate sufficient reserves for three ecdyses and all the nematode's metabolic needs until migratory females establish a feeding site. This non-feeding period lasts about 20 days at 25°C in sugarbeet (Inserra et al., 1983) or may be shorter at low population levels or in the presence of abundant roots. Putative quiescent capabilities of J3 and J4 could affect the frequency of migratory females in the population, minimizing competition for suitable sites for induction of syncytia.

In addition to *N. aberrans,* a number of other nematode species reportedly have active, non-feeding stages that may molt into feeding stages. Without feeding, J2 of *Rotylenchulus reniformis* Linford & Oliveira hatch and develop throughout three successive molts into immature females (Siddiqi, 1972). In Mermithidae, the first juvenile stages feed upon insect hemolymph, but the last juvenile stage and adults do not feed and, instead, live upon reserves stored in the trophosome. These reserves drive the last molt, gametogenesis, and mating (Batson, 1979). In *Paratylenchus bukowinensis* Micoletzky, J2, J3, and females are active feeders, but J4 are able to overwinter for 4 months and molt without feeding. Non-feeding J4 and males are found in other *Paratylenchus* species, and non-feeding J3 are found in *Gracilacus* spp. (Brzeski, 1976, 1995; Rhoades and Linford, 1961). Males of several Tylenchida, including *Radopholus* spp. and Criconematidae, presumably do not feed because they have a degenerate stylet and esophagus.

The study of esophageal glands in *N. aberrans* young and mature sedentary females reveals a pattern shared with heteroderids, including evidence of high secretory activity in the DG. This high activity suggests association of this gland with maintenance of syncytia, and perhaps with external and/or (or) internal digestion. Superficially, the SvG of *N. aberrans* sedentary females also resemble SvG of heteroderid females, with respect to their reduced size and apparent cessation of secretory granule production. Our TEM study revealed depleted secretory granules in the SvG ampullae, many secretory granules being recycled in large multivesicular bodies, and a complete set of organelles, including well-structured nuclei and nucleoli. SvG in young and mature sedentary females, two developmental phases separated by about 10 days (Inserra et al., 1983), seemed to have the same level of metabolic activity. We suggest that in sedentary females of *N. aberrans,* and perhaps also in heteroderids, SvG could have a function in digestion, but they also might function in other physiological processes that do not require massive production of secretory granules. This hypothesis, first formulated by Bird and Saurer (1967), requires further testing, specifically because involvement of esophageal glands in ecdysis has not yet been established (Bird, 1990; Singh and Sulston, 1978). In small and complex metazoans, including nematodes, a multiplicity of functions performed by some key organs might be necessary for evolutionary success. For example, the intestine of *Caenorhabditis elegans* (Maupas) Dougherty functions in digestion, and it is also the major site of synthesis of "yolk" proteins, a group of proteins that are exported to gonads to become the major protein component of oocytes (Kimble and Sharrock, 1983).

Ultrastructural and morphological changes in esophageal glands, correlated with developmental phases in the life cycle, provide a basis for new hypotheses on gland biology in *N. aberrans.* These hypotheses will be tested further with appropriate methods

for detection of synthetic activity in glands. We further anticipate that some of the molecular tools currently used to understand the molecular events involved in induction and maintenance of syncytia and giant cells by cyst and root-knot nematodes (Sijmons et al., 1994) ultimately will be applied to *N. aberrans* and other pratylenchids.

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