

Workshop: “Biology of Ampullariidae”

Minireview

Pigmented corpuscles in the midgut gland of *Pomacea canaliculata* and other Neotropical apple-snails (Prosobranchia, Ampullariidae): A possible symbiotic association

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Pigmented intracellular corpuscles (“spherioles”) have been known to occur in the midgut gland of ampullariid snails for many years (Meenakshi, 1955; Andrews, 1965; Lutfy and Demian, 1968).

Andrews (1965) described them in her morphological account of the midgut gland of *P. canaliculata* (Lamarck, 1822), and referred to them as “greenish spherules” and “brown concretions” (in this paper, they will be referred to as C and K corpuscles, respectively). She also ascribed a digestive-excretory function to C corpuscles and an excretory function to K corpuscles, since she found them associated with two distinct cell types in the gland acini: C corpuscles to the supposedly digestive (columnar) cells, and K corpuscles to the supposedly excretory (triangular or pyramidal) cells.

However, we were intrigued by the “cellular” appearance of C corpuscles, and by the hard multilamellar structure of K corpuscles, that we could only dissolve in hot sodium hydroxide solutions and found that it contains proteins. Therefore, the fecal loss of these physiologically valuable material was intriguing, particularly if the snail’s need of an adequate nitrogen and carbon balances were considered. So, we envisaged the possibility that they could be indeed the vegetative and kystic forms, respectively, of a prokaryotic symbiont¹ that was living and reproducing within the glandular cells, and that was also eliminated to the external environment, where it could also undergo a free-living cycle. The results of our first efforts to test this hypothesis are presented here.

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¹ The terms “symbiont”, “symbiosis”, and related terminology have been used in many ways (see Sapp, 1994, for a comprehensive history of these terms and of symbiosis theory). In the current review they will be used in the widest possible sense, to refer to any instance in which two different organisms live in close association, disregarding whether both or only one of them benefit from living together. The definition will encompass also those cases in which an organism exploits the use of functional portions of a second one, as when functional algal chloroplasts are acquired by certain molluscs (Felbeck *et al.*, 1983).

Morphology and species distribution of the putative symbiotic elements

In *Pomacea*, C and K corpuscles are sometimes eliminated in the feces in heavily packed droppings (Fig. 1a; Albrecht *et al.*, 2001 a, b). The main component of these droppings are sheathed, brownish/greenish C corpuscles, about 13 μm in diameter, that can be seen in unstained preparations (Fig. 1b); the pale but pigmented “cytoplasm” of these corpuscles contains several darker zones of irregular size and shape (Fig. 1b and 1d to 1g). K corpuscles are dark brown and club shaped, about 35 μm in length and 13 μm in width (Fig. 1c). Both C and K corpuscles also appear mixed with undigested food remnants in “normal” fecal droppings.

These putative symbiotic elements have been found in the feces of adult individuals of all the populations of *P. canaliculata* that have been studied so far², as well as in the adults of other congeneric species (Fig. 1, d-f). C corpuscles are also being eliminated in the feces of newborn *P. canaliculata* in laboratory culture as early as 4-5 days after hatching, while K corpuscles do not appear in the feces until much later.

A limited attempt was made to envisage the distribution of the putative symbiotic elements within the Neotropical section of the family Ampullariidae³. *P. insularum*, a species usually included within the “*canaliculata* group”⁴ (e.g., Cazzaniga, 1987; Cazzaniga, this issue of BIOCELL) was found to eliminate also C and K corpuscles in the feces (Fig. 1a), although K corpuscles are sometimes larger than in *P. canaliculata*. As in *P. canaliculata*, C and K corpuscles of *P. insularum* are eliminated either in “pure”, heavily packed droppings

(Fig. 1a), composed almost exclusively by corpuscles, as well as intermingled with food remnants in “normal” fecal droppings.

The putative symbiotic elements have been also found in the feces of *P. scalaris*, a species not included within the *canaliculata* group, and in *Asolene pulchella*. *Asolene* is a brevisiphonate Neotropical genus, generally placed fairly apart from *Pomacea* in phylogenetic trees of the Ampullariidae (Bieler, 1993). C corpuscles in these species are quite similar to those found in *P. canaliculata* (Fig. 1, d-f).

We attempted similar observations in the feces of the single *Marisa* individual we could collect, but we were unsuccessful. Therefore, we decided to sacrifice the animal and prepare the midgut gland for light microscopy. Dark brown K corpuscles were conspicuous but more rounded than in *Pomacea* (Fig. 2a). The gland histology differed from that of *P. canaliculata* and *P. insularum* where the cells containing K corpuscles did not appear reaching the epithelial surface (Fig. 2b). Smaller and paler pigmented bodies can be also seen within the gland epithelium, but they are not so regular in size and shape and they appear unsheathed (Fig. 2a).

Summarizing, the putative symbiotic elements appear to be essentially similar in *P. canaliculata*, *P. insularum*, *P. scalaris* and *Asolene pulchella*. Our failure to detect their fecal elimination in a single *Marisa* individual has to be confirmed by further observations. However, the presence of pigmented corpuscles in the midgut gland of this species suggests the existence of an association with a symbiotic partner resembling those found in the other studied species of Ampullariidae. This putative symbiotic association may have occurred after one or several independent episodes of infection, early in the evolution of Neotropical Ampullariidae.

Cellular associations of the putative symbiotic elements

As it was already mentioned, C corpuscles in the midgut gland alveoli are contained within columnar cells, while K corpuscles are found within pyramidal cells in *P. canaliculata* (Andrews, 1965; Albrecht *et al.*, 2001a, b). Both corpuscle types occupy altogether a significant part of the gland volume (6-14%; Albrecht *et al.*, 2001) and mass (11-13%; Vega *et al.*, 2001; both in males and females, respectively).

Usually, C corpuscles with a clearly visible sheath and a pigmented content, can be seen within the basal part of columnar cells, sometimes close to the basal membrane (Fig. 1i).

² Most of our studies on *P. canaliculata* were made on individuals collected in the Rosedal Lake (Palermo Park, Buenos Aires, Argentina). But other populations of this species were also sampled, most of them in Mendoza province: lakes of the San Martín Park (Mendoza City), Scaiola lake (Lavalle), Benegas dam (Rivadavia), El Carrizal dam (Luján de Cuyo) and one in Buenos Aires province, El Durazno creek (Gral. Alvarado).

³ For this purpose, species of three out of the five Neotropical genera recognized by Bieler (1993) were sampled. Several individuals of *P. scalaris* and *P. insularum*, and of *Asolene pulchella*, as well as one individual of *Marisa cornuarietis* were collected on the banks of the Paraná River, between the cities of Santa Fe and Santo Tomé (Argentina). *P. scalaris* can be easily distinguished from *P. canaliculata* because of the stair-stepped appearance of the shell. All of the shells of *P. insularum* individuals were longer than 70 mm, to avoid misdiagnosis with *P. canaliculata*, that could occur if smaller individuals were used. The shell shape and the siphon offered no difficulty for the diagnosis of *Asolene pulchella* and *Marisa cornuarietis*.

⁴ This group, proposed as the supraspecies *canaliculata* by this author, includes *insularum*, *haustum*, *lineata*, *sordida*, *dolioides*, *gigas*, *maculata*, etc.

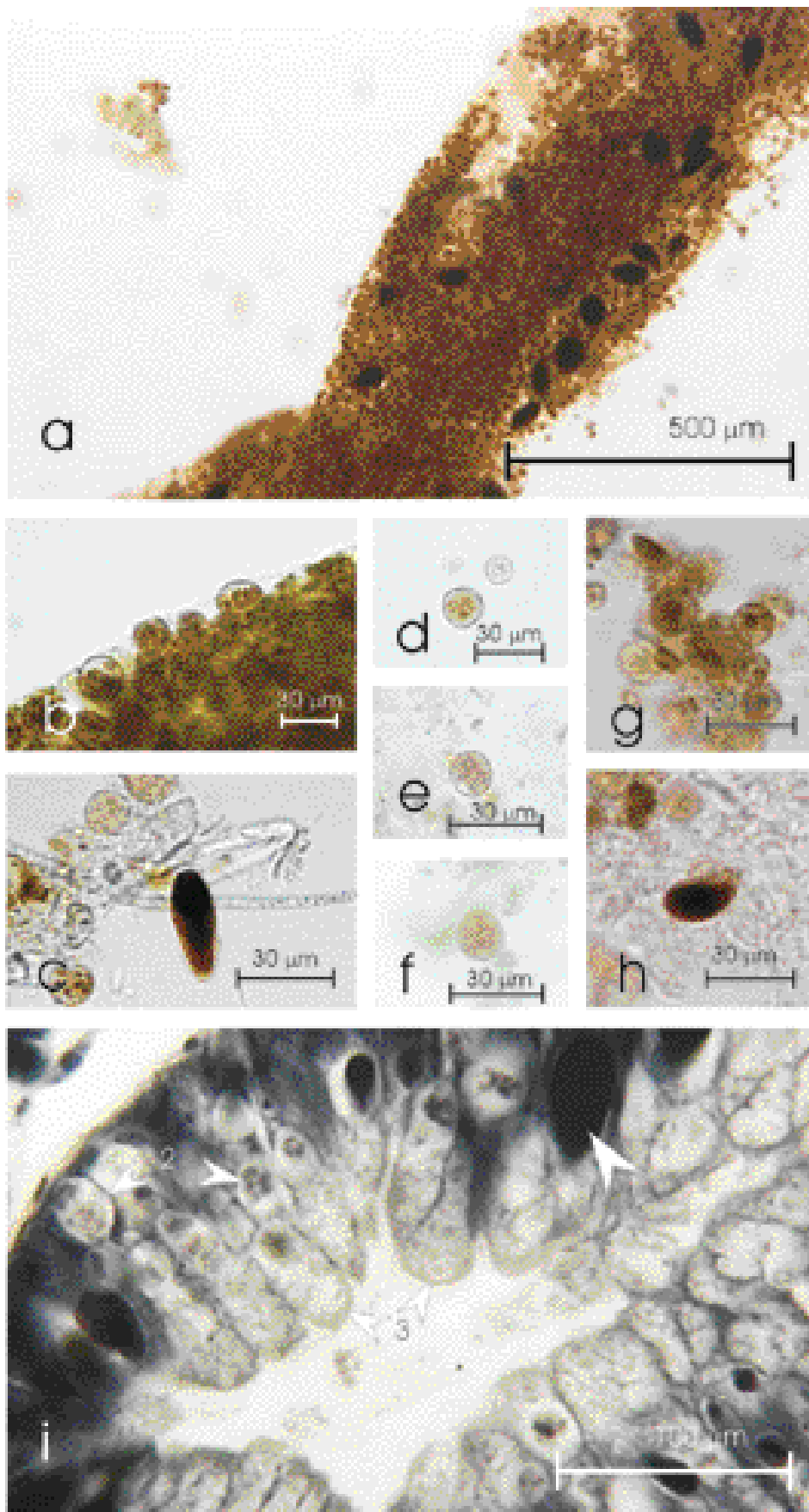


FIGURE 1. Panels *a* to *h* are micrographs from unstained preparations showing C and K corpuscles in fecal material from some Neotropical ampullariid species. Panel *i* shows an iron hematoxylin preparation of the midgut gland of *Pomacea canaliculata*. (*a*) fecal dropping of *Pomacea insularum* composed by a translucent matrix and the putative symbiotic corpuscles, i.e., the more abundant C corpuscles, and the larger and darker K corpuscles. (*b*) C corpuscles near the surface of a similar dropping of *P. canaliculata*; the darker zones within the cytoplasm of C corpuscles are easily distinguished in this, as well as in Figs. 1c-1g. (*c*) a K corpuscle in the feces of *P. canaliculata*. (*d*) C corpuscles in the feces of *P. insularum*; (*e*) idem for *P. scalaris*; (*f*) idem for *Asolene pulchella*. (*g*) C corpuscles in aquarium sediment, 13 months after removal of the snails. (*h*) a K corpuscle in the same sediment. (*i*) an alveolus in the midgut gland of *P. canaliculata* showing K corpuscles [1], sheathed C corpuscles in the basal third of columnar cells [2], and the "vacuoles" in the upper, dome shaped end of these cells [3].

Besides those basal, sheathed C corpuscles, the middle and apical regions of columnar cells appear full of vacuoles containing an unpigmented (or faintly pigmented) material that stains with iron hematoxylin. When these apical structures are observed under the electron microscope (Fig. 3, upper panel) they appear containing both small and large granules, and an irregular array of inner membranes. However, no membrane/granular associations such as grana or rough endoplasmic reticulum are observed. No nuclear structures are found either. Interestingly, a double external membrane can be frequently recognized (Fig. 3, upper panel, inset), which strongly suggests the symbiotic nature of these bodies.

These unsheathed corpuscles sometimes appear as either fusing or, more probably, dividing by constric-

tion (Albrecht *et al.*, 2001b). These apical structures can be seen when they are extruded from the cell into the lumen of the gland (Fig. 1*i*) as if a kind of apocrine secretion was going on. We believe these are the precursors of the pigmented and sheathed C corpuscles which appear later in massive amounts in the feces. In fact, the amount of sheathed and pigmented C corpuscles in the gland is far less than the amount of K corpuscles, while in the feces an inverse relation is observed. Therefore, what we consider the nude forms of C corpuscles, which are freed in the lumen of the gland, may develop the sheath and the brownish/greenish pigment during their passage through the gut.

Similarly vacuolated or granular upper ends of the epithelial cells can be seen in *Marisa cornuarietis*, and also, large (about 30 μm in diameter, Fig. 2, lower panel

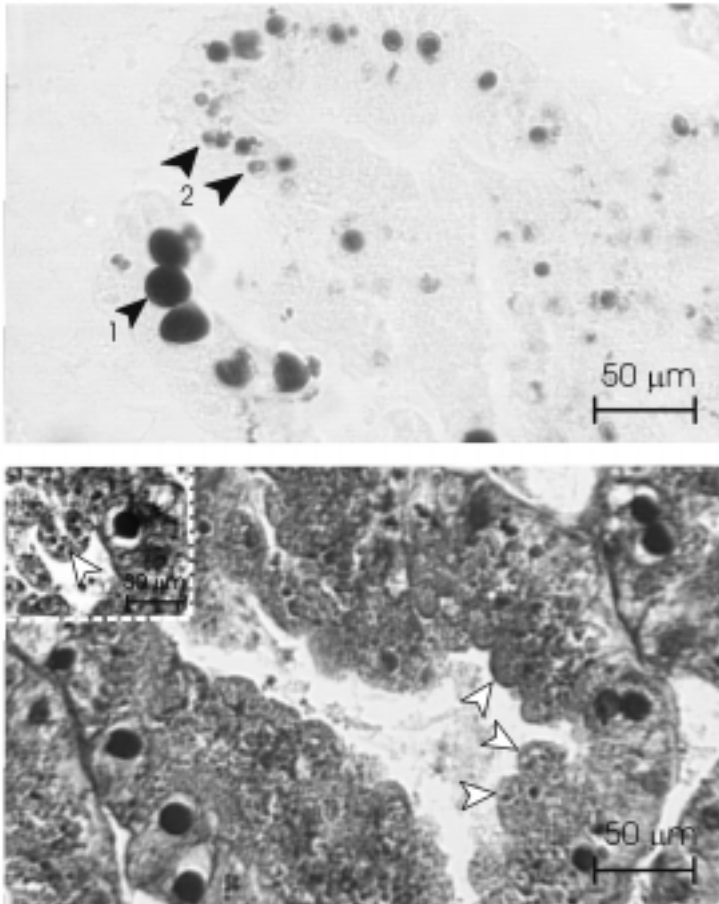
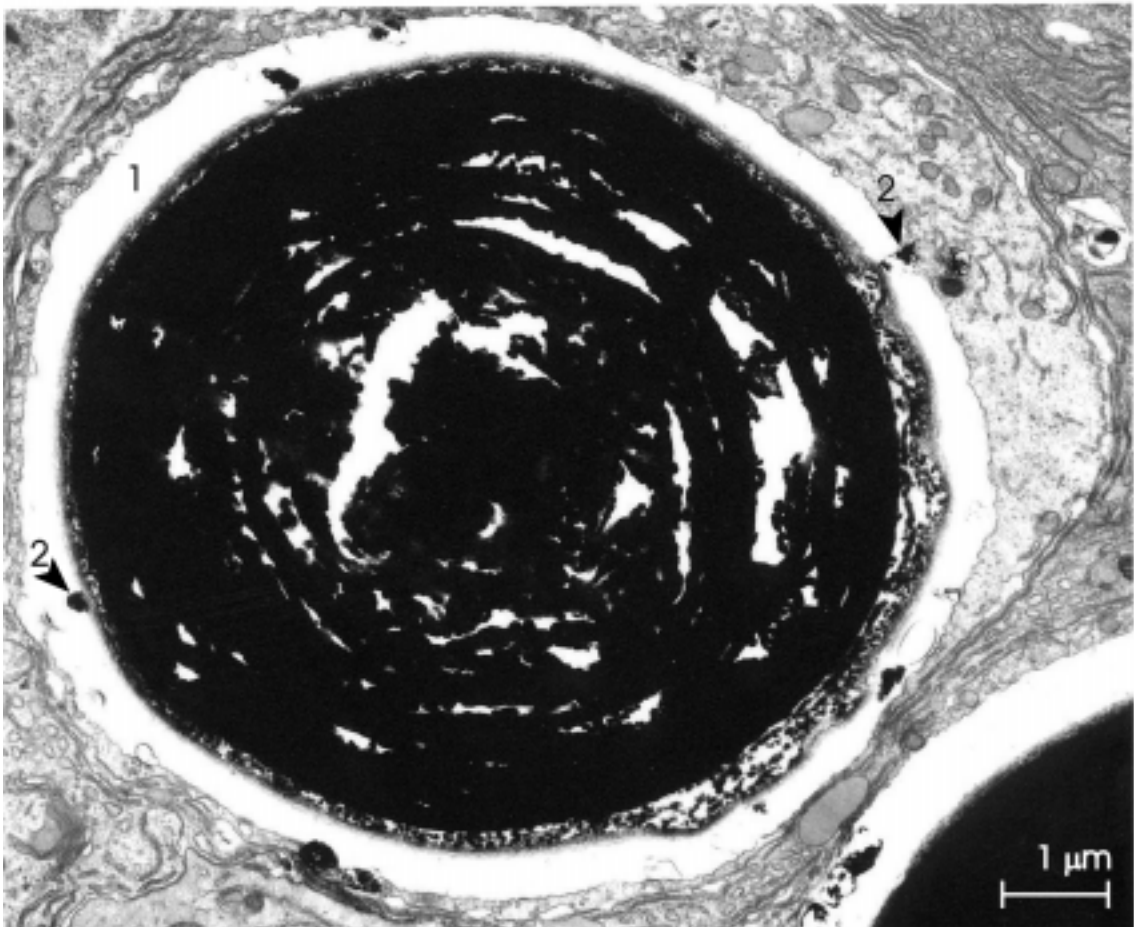
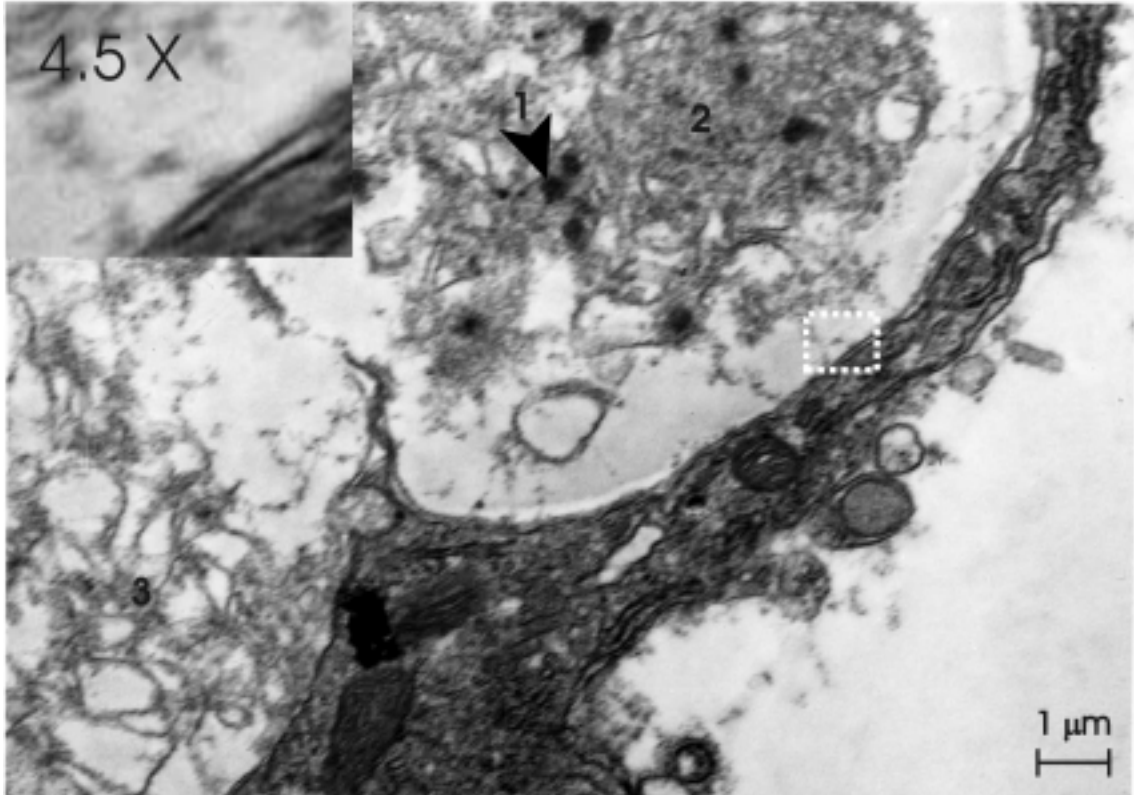


FIGURE 2. Micrographs of an alveolus of the midgut gland of *Marisa cornuarietis*. The upper micrograph is of an unstained section, where the rounded, well delimited K corpuscles can be seen [1], together with several smaller and irregular pigmented corpuscles [2]. The lower micrograph shows an hematoxylin-eosin preparation of the same gland, where the upper granular vacuolated ends of columnar cells are seen [1]. The inset shows a rounded granular vacuolated body, being freed in the lumen of the gland [arrow].

FIGURE 3. Upper panel: electron micrograph of a structure in the upper “vacuolated” end of columnar cells in *P. canaliculata*. This body, probably an unsheathed form of C corpuscles, contains large [1] and small [2] granules, and an irregular array of inner membranes [3]; it is also lined by a double membrane (as it can be better seen in the inset, which has been further enlarged). Lower panel: electron micrograph of a K corpuscle showing its multilamellar structure, and a membrane-lined surrounding space [1]; pieces of an electron dense material [2] appear crossing this space, from the surrounding cytoplasm to the peripheral part of the K corpuscle.



and inset) and unpigmented globular bodies (whose content is stained with iron hematoxylin) can be seen when they are freed from the epithelial cells into the lumen of the alveoli and into the excretory ducts.

Multilamellar K corpuscles are formed within the basophilic pyramidal cells. These cells show an important development of the rough endoplasmic reticulum under the electron microscope, which may be involved in synthesizing the proteins contained in the multilamellar coat. K corpuscles are later extruded into the lumen of the gland in *P. canaliculata*. We have posed the question of whether they are the kystic forms of C corpuscles, although we have no hint to say yet how a C corpuscle contained within a columnar cell may pass to a pyramidal cell to become a kyst.

Observations on C and K corpuscles in aquarium sediments and after isolation from gland homogenates

Microscopically normal C and K corpuscles could be observed in sediments of aquaria that had contained *P. canaliculata*, up to 13 months after removal of the snails (Fig. 1h), which indicates that the putative symbiont may also live outside the snail.

The isolation of these free-living C corpuscles in axenic cultures would have been instrumental to disclose the nature of these corpuscles. So far, however, we have been unsuccessful in this respect. So, a method to isolate C and K corpuscles from the midgut gland appeared important for studies on the nature of these putative symbiotic elements. Such a method was developed (Vega *et al.*, 2001a and b) and is been used to test some important predictions derived from the hypoth-

esis of the symbiotic nature of these corpuscles.

One of these predictions was that the corpuscles would have significant quantities of DNA, as any other living being. Confirming this view (Vega *et al.*, 2001a), DNA could be detected⁵ in fraction C in a concentration of 44.7 ± 9.2 $\mu\text{g}/\text{mg}$ of protein (mean \pm SEM, $n=7$). These values were intermediate to those found in *Escherichia coli* cells (64.9 ± 6.1 $\mu\text{g}/\text{mg}$, $n=9$) and human leucocytes (19.7 ± 2.9 $\mu\text{g}/\text{mg}$, $n=6$). DNA concentration in fraction K was 1.6 ± 0.4 $\mu\text{g}/\text{mg}$ ($n=12$), i.e., it was much lower than that found in fraction C. Those low values may be related to the high protein content of K lysates, and not to contamination of fraction K with C corpuscles (which is less than 1%); also, there are indications that a substance present in K lysates interferes with DNA measurements, and that DNA concentration may be actually higher than indicated above⁶.

Many other studies, as varied as the identification of pigments or the utilization of different substrates by the corpuscles, may take advantage of the possibility of studying the isolated corpuscles. As an example, Fig. 4 shows the absorption spectrum of a crude acetone extract of C corpuscles obtained from either lettuce - or paper-fed animals, which reminds the absorption spectrum of chlorophylls. Further studies will be needed to characterize the pigment/s present in the putative symbiotic elements as well as their possible function. However, their essentially similar spectra in corpuscles obtained from lettuce- and paper-fed animals suggest that the pigments are not coming directly from ingested photosynthetic cells.

Nitrogen excretion, nitrogen balance and the fecal elimination of the putative symbiotic elements in adult *P. canaliculata*

The midgut gland of prosobranchs, and the brown-greenish "spherioles" that originate in it, have long been thought to have an excretory function (Meenakshi, 1955; Andrews, 1965, 1981; Lutfy and Demian, 1968; Little, 1981). Since the fecal elimination of C and K corpuscles appeared important when assessed morphologically in *P. canaliculata*, we attempted to quantify the balance

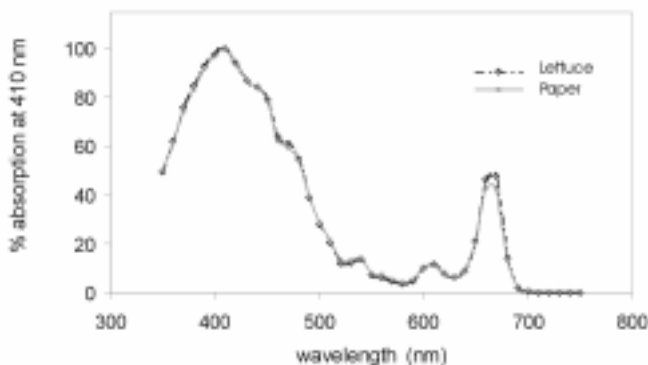


FIGURE 4. Light absorption spectra of acetone extracts of C corpuscles. The spectra of the pigment extracted both from lettuce - and paper-fed animals (*P. canaliculata*) are essentially the same, and are similar to those of chlorophylls.

⁵ The corpuscles were lysed by incubation in sodium hydroxide 0.2 N at 50°C for 25 min. DNA content was measured by the *bis*-benzamide method of Labarca and Paigen (1980) while the protein content was measured by the method of Lowry *et al.* (1951).

⁶ It appears that some substance present in K lysates, which is not DNA but may be bound to it, quenches the fluorescence of the *bis*-benzamide/DNA complex. Moreover, determination of DNA in lysates of the K fraction by the diphenylamine method (Burton, 1956) gave much higher concentrations than with the *bis*-benzamide method.

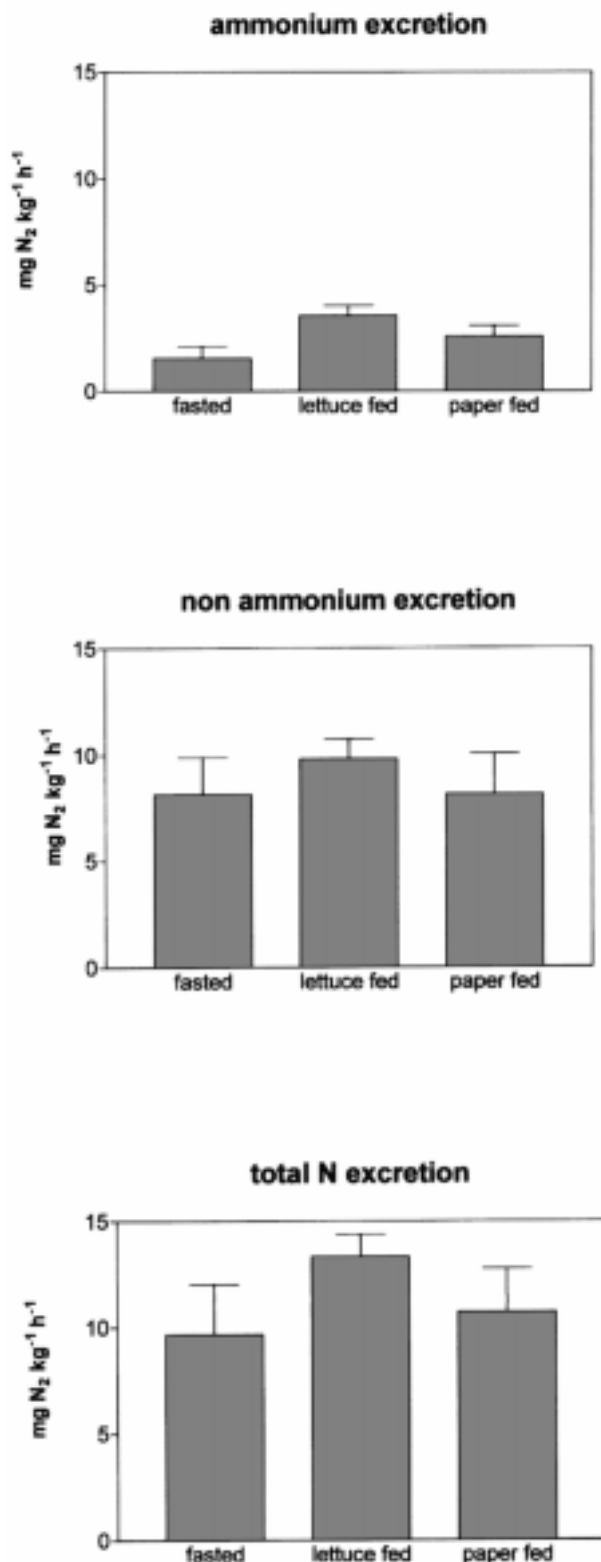


FIGURE 5. Forms of nitrogen excretion (ammonium excretion, non-ammonium substances and total combined nitrogen) in adult *P. canaliculata*, that were either fasted or fed with lettuce or paper during the last 48 h. The amount of ammonium nitrogen excreted was significantly higher in lettuce-fed animals (as compared with fasted or paper-fed animals, ANOVA I, Tukey test, $P < 0.05$). Other differences were not significant.

between ingested and excreted nitrogen in summer active snails.

We separately measured ammonium nitrogen excretion (the main nitrogen compound excreted in aquatic invertebrates, Schmidt-Nielsen, 1976) and the amount of combined nitrogen present in non-ammonium compounds (presumably associated to “excreted” corpuscles). We also wanted to assess if both the ammonium and non-ammonium nitrogen excretion varied if the snails were either fasted or fed *ad libitum* with either lettuce or paper⁷.

Surprisingly, only one third to one fifth of the total nitrogen excreted was in the form of ammonium. As expected, the amount of ammonium nitrogen excreted was significantly higher in lettuce-fed animals, as compared with fasted or paper-fed animals (Fig. 5). However the amount of non-ammonium nitrogen excreted (presumably associated to the putative symbiotic elements) did not varied significantly whether the animals were either fasted or not. When the balance between ingested and excreted nitrogen was estimated from the values in Fig. 5 and from the nitrogen content in food, the values were clearly positive for lettuce fed animals, while were negative for fasted and paper fed animals (Koch *et al.*, 2001). It seems therefore that although the fecal loss of nitrogen associated to the putative symbiotic elements is important, it may be thoroughly compensated by the combined nitrogen contained in a lettuce diet.

Context and perspectives

And excretory function has been attributed to the pigmented corpuscles in the midgut gland of ampullariids for decades (Meenakshi, 1955; Andrews, 1965; Lutfy and Demian, 1968). However, we have reviewed here the evidence suggesting the symbiotic nature of the “spherioles” present in *P. canaliculata* and other Neotropical Ampullariidae. The emerging picture suggests

⁷ Adult active snails of both sexes, obtained from the Rosedal Lake (Palermo, Buenos Aires) and maintained in external ponds during the summer months, were fed with a mixed vegetable chow. When needed, groups of these individuals were transferred to an air conditioned room (22°C) and they were either fasted for 48 h or fed *ad libitum* with nitrogen-free paper or with lettuce (they consume daily an amount of lettuce equal to 17% of their biomass, Albrecht *et al.*, 1999). Afterwards, groups of animals were put in an aquarium with tap water and no food (approx. 0.3 kg of wet biomass per liter). Two hours later, both water and sediments were collected, thoroughly dispersed and sampled. Ammonium nitrogen was measured by distillation of the alkalized samples. Combined nitrogen in the remaining dry residue was measured by the Kjeldahl method. Nitrogen values detected in tap water were subtracted from all measurements and the amount of combined nitrogen was calculated as mg N/ kg biomass/ h.

that C corpuscles are indeed the vegetative forms of a prokaryotic symbiont inhabiting the columnar cells of the midgut gland alveoli. The possibility that K corpuscles were the kystic forms of the same organism should be also considered.

Many species of mollusks are known to participate in symbiotic associations with either algal cells and chloroplasts, or with bacterial cells (Felbeck *et al.*, 1983). The organs involved may be either the mantle (e.g., Trench *et al.*, 1981), the gills (e.g., Windoffer and Giere, 1997), the gut (e.g., Rosenberg and Breiter, 1969) or the midgut gland (e.g., Griebel, 1993).

The latter association is known in *Elysia viridis* and other opisthobranch mollusks which are specialized grazers feeding on one or a few algal species, and which incorporate the algal chloroplasts into the cells of the midgut gland (Taylor, 1968; Graves *et al.*, 1979; Griebel, 1993). Because of the size of C corpuscles and their location in the midgut gland cells of *P. canaliculata*, this putative symbiotic association reminds somehow this case. Chloroplasts in the midgut gland of opisthobranchs are able to function photosynthetically, since the many terminal glandular tubules are located below the slug's dorsal mantle and provide a substantial surface for light absorption. It is known that mucus synthesis in these opisthobranchs is largely supported by photosynthetically derived sugars from the hosted chloroplasts (Trench *et al.*, 1974; Felbeck *et al.*, 1983).

However, there are some aspects in which the association with *P. canaliculata* and that with *E. viridis* differ:

(1) although C corpuscles in *P. canaliculata* seem to contain a chlorophyll-like pigment, the structure of the midgut gland in *P. canaliculata* does not provide a surface exposed to light as in *E. viridis*. Indeed, the midgut

gland alveoli where the C corpuscles are located is covered by a darkly pigmented epithelium (Albrecht and Cavicchia, 2000), the shell and the periostracum.

(2) though C corpuscles show some inner membrane structures, they do not show typical chloroplast structures such as grana.

(3) the possibility of an alimentary origin of C corpuscles seems to be ruled out by their persistence in the midgut gland and feces of adult snails feeding only on paper for periods of up to 2 months (Vega and Castro-Vazquez, unpublished), and by their appearance in hatchlings cultured in sterile media in which growing of any photosynthetic organism was prevented (Koch and Castro-Vazquez, unpublished).

The putative symbiotic elements have been found in all *P. canaliculata* individuals that we have sampled so far, so we believe that they are a normal constituent of the midgut gland of this species. Therefore, one may wonder which might be the physiological advantage (for the apple-snail) of carrying a lodger that occupies a significant part of its most voluminous organ, and which causes significant fecal losses of both nitrogen and carbon. In general, the benefits reaped by mollusks that live in symbiotic associations are nutritional ones, and therefore, it is reasonable to hypothesize that ampullariids may compensate for the nitrogen and carbon losses through some unknown nutritional advantage. An interesting possibility that should be explored is that the current association were a chemosymbiosis, that would take advantage of the usually high sulfide content of sediments where these snails live. Such chemoautotrophic associations have been described in deep sea mollusks that live in sulfide-rich hydrothermal vent environments (Cavanaugh, 1994; Felbeck, 1987; Shively *et al.*, 1998; Windoffer and Giere, 1997).

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