

Evidence for maternal transmission of a putative endosymbiont in the digestive gland of *Pomacea canaliculata* (Architaenioglossa, Ampullariidae)

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Abstract: The digestive gland of the apple snail *Pomacea canaliculata* lodges two types of pigmented corpuscles (identified as C and K corpuscles) which has been proposed as endosymbiont/s. Both corpuscular types are always present in the digestive gland of adult snails, they are released into the tubuloacinar lumen and are later expelled in the feces. On their part, hatchlings lack any C or K corpuscles in the digestive gland as well as in their feces, whereas C corpuscles appear in both the gland and feces within one week after hatching. Hence, it is possible that the detritivorous hatchlings acquire the putative C-endosymbiont from feces in the sediments where they live, i.e. through 'lateral' or 'horizontal' transmission. This possibility was put to test in an experiment in which we prevented any lateral transmission, by a 7-days aseptic culture, with no food, of aseptically obtained hatchlings. At the end of the experiment, we observed that most juveniles had survived the culture period, and hence the digestive glands and feces of survivors were studied by light microscopy of resin embedded, toluidine blue-stained sections. All studied glands and fecal samples showed C corpuscles. It is concluded that lateral transmission of the endosymbiont, if any, is not indispensable for the acquisition of the endosymbiont by hatchlings.

There is an ample, though fragmentary, evidence indicating that two types of pigmented corpuscles (identified as C and K corpuscles, Castro-Vazquez *et al.*, 2002) are present within specific cells of the digestive gland and/or in feces of adult Ampullariidae, comprising species of the African clade of this family (*Lanistes* and *Pila*, Ademolu and Castro-Vazquez, unpublished data), the Asian clade (*Pila*) (Meenakshi, 1955; Devi *et al.*, 1981; Takebayashi, 2013), and of the Neotropical *Marisa* and *Pomacea* clades (Castro-Vazquez *et al.*, 2002; Takebayashi, 2013). All these clades are named according to Hayes *et al.* (2009) and encompass all the extant ampullariid genera, with the single exclusion of the monotypic African genus *Afropomus*.

These corpuscles are lodged within specific cells of the tubuloacinar digestive gland (Castro-Vazquez *et al.*, 2002; Koch *et al.*, 2006) and it has been suggested they are endosymbionts related to Cyanobacteria (Castro-Vazquez *et al.*, 2002; Vega *et al.*, 2006; Dellagnola *et al.*, submitted) and/or chloroplasts (Vega *et al.*, 2012).

Both types of pigmented corpuscles are present in the gland and/or feces of all populations of *P. canaliculata* that have been studied so far, which strongly suggests that this putative endosymbiosis would be obligate for the snail host (Castro-Vazquez *et al.*, 2002; Vega *et al.*, 2006).

Both corpuscular types are released from the gland into the gut lumen and later expelled in the feces (Castro-Vazquez *et al.*, 2002). Also, C but not K corpuscles remained for more than 3 years in sediments of aquaria that have contained *P. canaliculata* (Koch *et al.*, 2006) and it is possible, therefore, that C corpuscles undergo part of their life cycle in the environment.

No pigmented corpuscles are present in the digestive gland in pre-hatching juveniles of *P. canaliculata* (Koch *et al.*, 2009) nevertheless C corpuscles appear in the digestive gland and feces within one week after hatching. On their part, K corpuscles appear two weeks after hatching (Koch, 2008). The present note will be focused on C corpuscles only.

The detritivory of hatchlings suggested us that the putative endosymbiont was acquired by consumption of C corpuscles in the environment, particularly from fecal droppings of adult snails, i.e. that the endosymbiont was

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maintained in the snail's populations through 'lateral' or 'horizontal' transmission. However, results reported here indicate that exclusion of lateral transmission does not prevent the appearance of C corpuscles at the expected time, which suggest a kind of 'vertical' or 'maternal' way of transmission in the host populations.

In this experiment, eggs in recently laid clutches were dispersed by gentle shaking in a 0.2 N NaOH+1% sodium dodecyl sulfate solution. Then, each egg was washed repeatedly with sterile buffer (0.06% NaCl and 100 mM phosphate buffer; pH=7.4), dried in a laminar flow cabinet within sterile Petri dishes, and then incubated at 26°C. By the time the eggs were about to hatch (13-14 days after oviposition, Koch *et al.*, 2009). Previous to extraction of the prehatching juveniles, the egg surface was sprayed with a povidone-iodine solution (Pervinox[®]) to ensure sterility. Nevertheless, the capsule was carefully opened with sterile tweezers, avoiding any contact between the prehatching juvenile and the external surface of the capsule.

Juveniles thus obtained were cultured for 7 days in 14 flasks, each one containing 5 juveniles and 10 mL of an autoclaved culture medium (the supernatant of recently laid egg clutches homogenized in 0.06% NaCl, and centrifuged at 750 g for 10 min). Streptomycin (10 µg/mL) and penicillin (480 UI/mL) were added to the medium before use. Control juveniles were similarly obtained, but were cultured in clean but not aseptic aquaria, and were fed lettuce. Samples of the digestive gland were obtained from both experimental (N=15) and control juveniles (N=10) and were fixed in Karnovsky's fluid, embedded in Spurr's resin, and 1 µm sections were obtained in an ultramicrotome and stained with toluidine blue. Two out of 14 flasks showed bacterial contamination and were discarded from the study. Also, samples of the digestive gland of the aseptically obtained hatchlings were fixed and processed for microscopy.

On the day when the hatchlings were aseptically obtained, the digestive gland were composed of round acini, with interspersed with regressing, giant albumen cells. The round acini were composed of two cell types that can be correlated with the pyramidal and columnar cells found in adult snails (Koch *et al.*, 2006), even though they were smaller and lacked the associated C or K corpuscles (Fig. 1). Pyramidal cells showed a pale cytoplasm and their nuclei were large and exhibited 1-3 conspicuous nucleoli. Columnar cells were loaded with endocytosed albumen (heavily stained with toluidine blue) and their smaller nuclei were seldom seen, and showed a single, inconspicuous nucleolus. Large extracellular albumen masses were also seen, both within and between the acini, which are remnants of the albumen that filled the midgut during preceding developmental stages (Koch *et al.*, 2009).

Most juveniles that were aseptically obtained survived to day 7, whether they were cultured in standard aquarium conditions and fed with lettuce, or in sterile flasks containing the nutrients-rich culture medium. The digestive gland of juveniles under both treatments had changed dramatically by day 7, and there were not appreciable differences between them (Figs. 2A and 2B). All juveniles showed C corpuscles contained within large vesicles in cells of the digestive gland. Even though the host's cell nuclei had enlarged only slightly, the cytoplasm of both pyramidal and columnar cells had markedly enlarged, and the formerly round acini had become elongated tubuloacini, as those of adult snails (Koch *et al.*, 2006). In contrast to the observations on day 0, the supranuclear cytoplasm of columnar cells was loaded with clear and small endocytic vesicles and, in general, the cytoplasm of pyramidal cells was darker than that of columnar cells. K corpuscles were not seen. Groups of smaller and darkly stained clumps that were contained within vesicles were also seen, but were more frequent and darker in juveniles cultured in aquarium conditions (Fig. 2A).

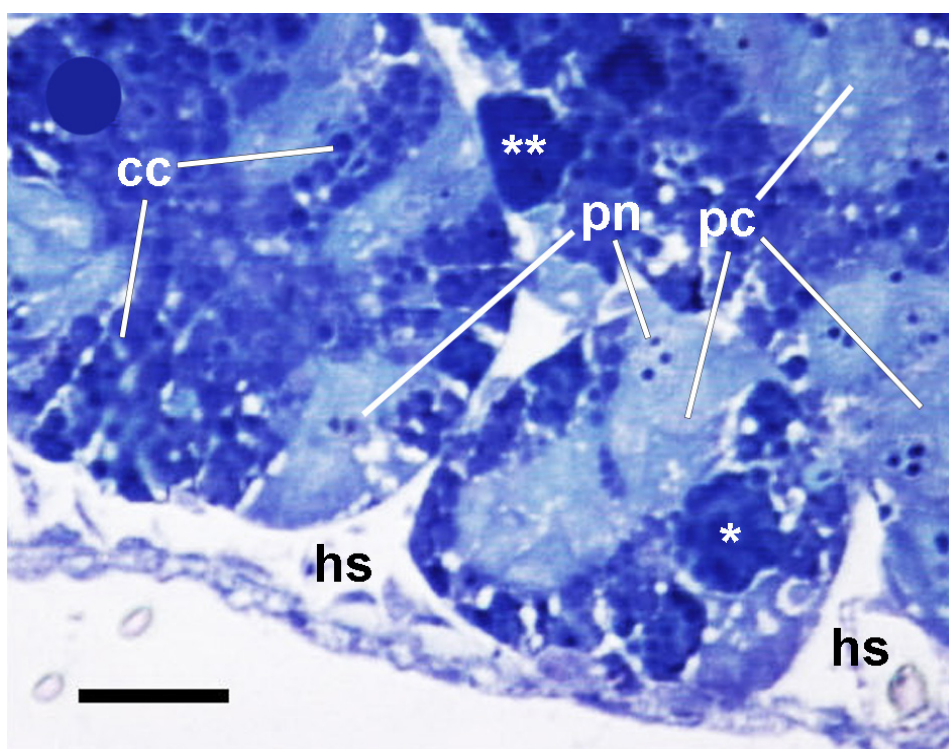


FIGURE 1. Developing digestive gland acini of a 0-days old juvenile showing no endosymbiotic corpuscles. Columnar cells show massive albumen endocytosis (dark blue) and their nuclei are small and difficult to discern. The cytoplasm of pyramidal cells appears pale blue and their nuclei are large and bear 1-3 conspicuous nucleoli. Large extracellular albumen masses (dark blue) are also seen, either within (*) or between (**) the developing acini. Abbreviations: cc, columnar cells; hs, hemocoelic space; pc, pyramidal cell cytoplasm; pn, pyramidal cell nucleus. Scale bar: 10 µm.

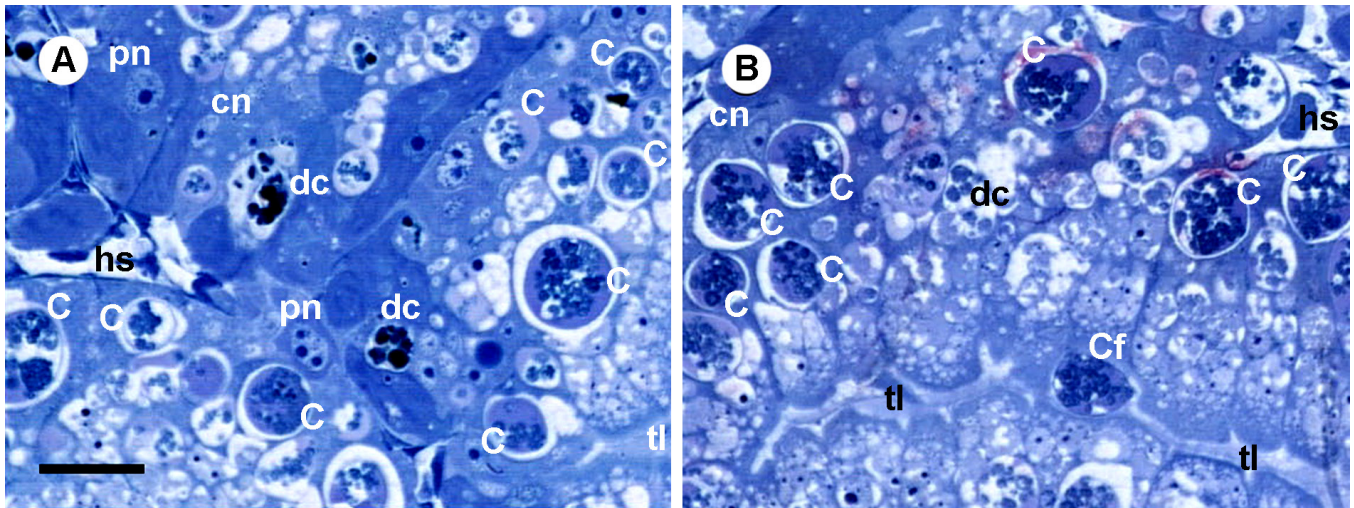


FIGURE 2. Digestive gland sections of 7-days old juveniles showing numerous C corpuscles contained within glandular cells, both in juveniles cultured in standard aquarium conditions (A) and in sterile flasks (B). A C corpuscle is seen after exocytosis towards the tubuloacinar lumen in panel B. Abbreviations: C, intracellular C corpuscle; Cf, C corpuscle free in the tubuloacinar lumen; cn, columnar cell nucleus; dc, dark clumps; hs, hemocoelic space; pn, pyramidal cell nucleus; tl, tubuloacinar lumen. Scale bar=10 μ m.

The universal occurrence of pigmented corpuscles in populations of *P. canaliculata* (Castro-Vazquez *et al.*, 2002) and of other Ampullariidae (Dellagnola *et al.*, 2016) suggests that effective mode/s of transmission of the putative endosymbiont/s are at work. As mentioned above for *P. canaliculata*, a simple possibility was that the detritivorous hatchlings were acquiring the endosymbiont laterally, i.e. from adult fecal material in sediments. However, the experimental exclusion of that possibility did not prevent the appearance of C corpuscles in the digestive gland one week after hatching (Figs. 2A and 2B), i.e. at about the normal time of their appearance after hatching.

This strongly suggests that a form of direct maternal transmission occurs, and it opens the possibility that the putative endosymbiont had co-evolved with its host, which would explain the differences in the morphology of C corpuscles that are found in at least some Neotropical Ampullariidae (Dellagnola *et al.*, submitted).

This experiment also provides conclusive evidence against the hypothesis of C corpuscles being remnants of the intracellular digestion of chloroplasts, because they appeared in the digestive gland of juveniles that had not consumed any plant material.

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