The midgut ultrastructure of the endoparasite *Xenos vesparum* (Rossi) (Insecta, Strepsiptera) during post-embryonic development and stable carbon isotopic analyses of the nutrient uptake

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Abstract

Females of the endoparasite *Xenos vesparum* (Strepsiptera, Stylopidae) may survive for months inside the host *Polistes dominulus* (Hymenoptera, Vespidae). The midgut structure and function in larval instars and neotenic females has been studied by light and electron microscope and by stable carbon isotopic technique. The 1st instar larva utilizes the yolk material contained in the gut lumen, whereas the subsequent larval instars are actively involved in nutrient uptake from the wasp hemolymph and storage in the adipocytes. At the end of the 4th instar, the neotenic female extrudes with its anterior region from the host; the midgut progressively degenerates following an autophagic cell death program. First the midgut epithelial cells accumulate lamellar bodies and then expel their nuclei into the gut lumen; the remnant gut consists of a thin epithelium devoid of nuclei but still provided with intercellular junctions. We fed the parasitized wasps with sugar from different sources (beet or cane), characterized by their distinctive carbon isotope compositions, and measured the bulk $^{13}C/^{12}C$ ratios of both wasps and parasites. Female parasites developing inside the wasp hemocoel are able to absorb nutrients from the host but, after their extrusion, they stop incorporating nutrients and survive thanks to the adipocytes content.
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1. Introduction

The strepsipteran *Xenos vesparum* (Rossi) is an obligate endoparasitic insect with an extreme sexual dimorphism. The female is a neotenic larviform viviparous organism, permanently associated with the paper wasp *Polistes dominulus* (Christ). At sexual maturity it extrudes from the wasp abdomen with only its anterior region, the "cephalothorax". A ventral canal opens with a transversal fissure in the cephalothorax and connects the hemocoel to the exterior; through this opening, both insemination and release of triungulins occur (Hughes-Schrader, 1924;

Beani et al., 2005). The adult male is a winged short-living organism (<5 h) that, after having spent all its pre-imaginal instars as an endoparasite, emerges from its puparium just to inseminate a female (Kathirithamby, 1989; Beutel and Pohl, 2006). The male represents the only free-living organism together with the 1st instar larva (the so-called "triungulin"); this is the host-seeking stage, able to infect all larval stages of *P. dominulus*.

X. vesparum spends its larval life lying in the hemolymph of the host. If female, it may survive and overwinter inside female wasps (Hughes et al., 2004a). Next spring, these wasps—physiologically castrated, i.e. with undeveloped ovaries (Strambi and Strambi, 1973a)—will be not involved in colony activity (Beani, 2006), but will function as a vehicle of *X. vesparum* females and mobile dispensers of triungulins

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until summer. Thus, this continuous, intimate association of *X. vesparum* females with their hosts may last almost 1 year and, if male, about 2 months, because either wasp or parasite dies at the end of summer. Infected wasps are inactive (Hughes et al., 2004b; Dapporto et al., 2006). The nutrient balance of this long-term coexistence has to be finely tuned to the development of both host and parasite.

Strambi and Strambi (1973b) claimed that the larvae of *X. vesparum*, during their endoparasitic development in the wasp hemocoel, have a functional alimentary system and nutrients are ingested from the host hemolymph. By means of histo-autoradiographic analyses, the incorporation of labeled amino acids into the adipocytes, evident until the early 4th instar, was no longer observed after the extrusion of the neotenic females. This is in disagreement with the hypothesis of Nassonow of a nutrient uptake through the larval cuticle (see Imms, 1973) via osmosis (Jeannel, 1951). More recently, Kathirithamby (2000) proposed that in neotenic females of *Stichotrema dalla-torreanum* (Myrmecolacidae), nutrient uptake occurs via a modified ventral structure, the so-called *apron*. "This is the first case where in an endoparasitic insect an outer surface coat is analogous to the midgut, where both a matrix analogous to the peritrophic matrix and a brush border of microvilli occur". At least in this species, the ventral canal opening in cephalothorax would act "as an absorptive surface", as well as an escape route for triungulins (Kathirithamby, 2000).

The aim of this work, focused on *X. vesparum* (Stylopidae), is to establish whether the ultrastructure of the midgut cells supports the absorption of nutrients from host hemolymph, either during larval stages or in extruded neotenic females. Here microscope data are implemented by stable carbon isotopes analyses, which are commonly used to describe dietary differences in animals. As the whole body of an animal reflects the isotopic composition of its diet (De Niro and Epstein, 1978), the stable carbon isotopic technique provides a powerful tool to investigate animal dietary behavior (e.g. Gannes et al., 1998). Naturally occurring differences in the $^{13}C/^{12}C$ food ratios reflect the trophic preferences (Tieszen et al., 1979; Spain and Reddell, 1996; Spain and LeFeuvre, 1997; Chamberlain et al., 2004). In the present study, the different carbon isotope compositions of parasitized wasps, after an experimental diet turnover, were used to monitor the alimentary relationship between wasp and parasite: we questioned whether *X. vesparum* females, at a certain stage of their development, stopped ingesting and/or absorbing nutrients from the wasp hemolymph, according to a parasitic strategy of sustainable exploitation of host resources.

2. Materials and methods

2.1. Endoparasitic life cycle of X. vesparum

After its entry into the host, the 1st instar larva undergoes hypermetamorphosis (Kathirithamby, 1989) and gives rise to an apodous 2nd instar larva through a normal molting process. On the contrary, the following molts are characterized by "apolysis without ecdysis" (Kathirithamby et al., 1984): the

exuviae of the previous instars are retained to surround the whole body. In the female parasite, germ cell differentiation takes place in the 3rd instar (Kathirithamby et al., 1990), and sexual maturity is reached at the 4th instar, when the wasp is in its pupal stage or emerges from the nest cell. It grows hidden inside the wasp hemocoel until it extrudes its cephalothorax from the wasp tergites. The neotenic female lacks the morphological features of an adult insect, such as wings, legs, external genitalia, mouthparts. For the purposes of our study, we analyzed the midgut of the 1st to 3rd larval instars of both sexes, and 4th instar females before and after the extrusion of cephalothorax.

2.2. Study animals

The source of *X. vesparum* females was a set of *P. dominulus* naturally infected nests from the surroundings of Florence (Tuscany, Italy), and a set of artificially infected nests (for methods see Hughes et al., 2004b). To study the ultrastructure of the midgut, we used parasites of different ages. *X. vesparum* larvae were obtained from *Polistes* larvae and pupae of artificially infected nests, as well as unextruded *X. vesparum* females, hidden in the abdomen of "crypto-parasitized" wasps: i.e. wasps not yet visibly parasitized, but emerging from marked cells, previously infected with triungulins, and later found to be parasitized by one *X. vesparum* female. "Young" (summer sample, $N = 18$), "overwintering" (winter sample, $N = 15$) and "old" (post-hibernation sample, $N = 22$) fertilized *X. vesparum* females (extruded from 2 months, 5 months and 1 year, respectively) were dissected from visibly parasitized wasps which were kept in the laboratory in light/temperature natural conditions.

We carried out stable carbon isotopes analyses on wasps: "crypto-parasitized" ($N = 4$); visibly parasitized by one "young" *X. vesparum* female ($N = 15$); "control", i.e. not parasitized ($N = 6$). All these samples were maintained in $20 \times 20 \times 20$ cm plexiglas cages and fed with beet and/or cane sugar *ad libitum*.

2.3. Transmission electron microscopy (TEM)

Different larval stages (1st instar larvae $N = 28$; 2nd instar larvae $N = 24$; 3rd instar larvae $N = 22$; unextruded 4th instar larva $N = 8$) of *X. vesparum* were extracted from the host and, under a stereomicroscope, cut transversally in two pieces in 0.1 M, pH 7.2 phosphate buffer (PB) to which 1.8% sucrose had been added. We isolated, in the same buffer, the gut of the neotenic females of *X. vesparum* of different ages (see Section 2.2). Both samples were fixed for 24 h at 4 °C in 2.5% glutaraldehyde in PB. The material was then rinsed in buffer and post-fixed in 1% osmium tetroxide in PB for 1 h at 4 °C. After dehydration in a graded series of ethanol, the material was embedded in an Epon–Araldite mixture. Ultrathin sections, obtained with a Reichert Ultracut II E and an LKB Ultratome III, were routinely stained (1% uranyl acetate following by 1% lead citrate) and observed with a Philips CM 10 transmission electron microscope at 80 kV. Semithin

sections, of about 1 μm, were stained for 5 min with 1.5% toluidine blue and observed with a Leica DMRB light microscope.

2.4. Scanning electron microscopy (SEM)

For scanning observations, the samples were fixed as described above and rinsed several times in PB. After dehydration in ethanol, the material was processed by the critical point drying method in a Balzer's CPD 010 critical point dryer. The material was mounted on aluminum holders by a carbon conductive glue and sputtered with about 20 nm gold evaporated in a Balzer's MED 010 sputtering device. Samples were observed with a Philips XL20 scanning electron microscope operating at an accelerating voltage of 25 kV.

2.5. Preliminary assay of the cuticle permeability

For the preliminary assay of cuticle permeability, 0.05 ml of 1% methylene blue in PB was injected into the hemocoel of wasps ($N = 5$) infected by an extruded neotenic female. Three days after the injection, the wasps were dissected to remove both the wasp alimentary apparatus and the parasites. The samples were rinsed in PB and observed under a stereomicroscope.

2.6. Stable carbon isotopes analysis

Crypto-parasitized wasps ($N = 4$) were separated from those visibly parasitized by one female extruded from 2 months, i.e. "young" ($N = 15$). Specimens from these groups were differently fed with beet sugar and cane sugar, characterized by different $\delta^{13}C$ values (beet sugar $\delta^{13}C = -24.80$; cane sugar $\delta^{13}C = -10.87$).

Wasps parasitized by one female were fed only with beet sugar ($N = 5$; Exp. 1) or first fed with beet sugar and then with cane sugar for 3 days ($N = 5$; Exp. 2) or for 10 days ($N = 5$; Exp. 3). The crypto-parasitized wasps were fed only with cane sugar ($N = 2$; Exp. 4) or with beet sugar ($N = 2$; Exp. 5). For Exp. 2 and 3 we also used uninfected wasps as controls ($N = 6$).

At the end of these dietary regimes, tissues from wasps and parasites were rinsed several times in distilled water and lyophilized, to be analyzed for stable carbon by continuous flow isotope ratio mass spectrometry (CF-IRMS). Samples were wrapped in tin capsules and ash combusted at 1120 °C, followed by reduction at 640 °C using a ThermoFinnigan Carlo Erba Elemental analyzer. The CO_2 produced was purified by on-line removal of water and chromatographic separation, and introduced directly into the mass spectrometer (ThermoFinnigan Delta XP) via helium carrier gas. A pulse of CO_2 reference gas was introduced into the mass spectrometer with the CONFLOIII automated injection system, to carry out comparative analyses.

All the results are expressed in the conventional delta (δ) notation in parts per thousand:

$$\delta = \left(\frac{R_x - R_{std}}{R_{std}} \right) \times 1000$$

relative to Pee-Dee Belemnite. The analytical precision was better than 0.2%. The reported $\delta^{13}C$ values represent the average ($\pm$ SD) of three or more analyses, for each sample, of total animal carbon, as slight isotopic differences among distinct specimens may arise from minor sample heterogeneity. The standards used to calibrate the method were 0.1 mg aliquots of in-house graphite ($\delta^{13}C = -25.92 \pm 0.17‰$, $n = 10$), NIST std standards RM8542 (sucrose; $\delta^{13}C_{acc} = -10.47‰$; $\delta^{13}C_{meas} = -10.41 \pm 0.15‰$, $n = 7$) and RM8540 (polyethylene foil; $\delta^{13}C_{acc} = -31.77‰$; $\delta^{13}C_{meas} = -31.66 \pm 0.08‰$, $n = 4$). Commercial beet sugar ($\delta^{13}C = -24.80 \pm 0.01‰$, $n = 6$) and cane sugar ($\delta^{13}C = -10.87 \pm 0.01‰$, $n = 5$) were used to feed the wasps.

3. Results

3.1. Assay of the cuticle permeability by injection of methylene blue

This preliminary trial was performed on wasps parasitized by an extruded neotenic female. After injection of 1% methylene blue in PB in the wasp abdomen, the stain diffused through the basal lamina into the midgut epithelium, with a main storage of pigment in the posterior tract of the alimentary system and into the Malpighian tubules that appeared strongly stained. On the contrary, there was no trace of methylene blue at any level of the *X. vesparum* female body isolated from the wasp (Fig. 1A,B).
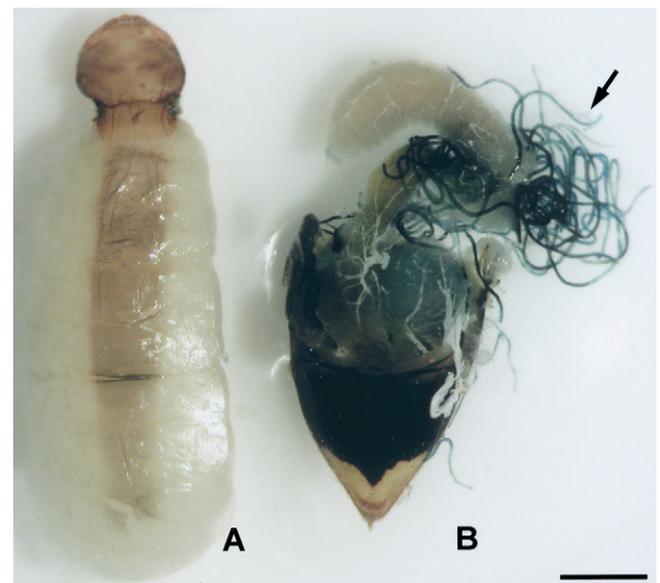

Fig. 1. After injection of methylene blue in the abdomen of a parasitized wasp, the isolated neotenic female (A) does not show any trace of the stain while the Malpighian tubules (arrow) of the wasp (B) have marked pigment. Scale bar 1 mm.

3.2. Midgut ultrastructure of the parasite during its larval instars

The 1st instar larva (Fig. 2A) has a monolayered midgut epithelium (Fig. 2B), about 2 μm high. It sits on a very thin basal lamina, beneath which muscle fibers are evident. The epithelial cells show: an elongated nucleus with compact masses of chromatin, a rich system of rough endoplasmic reticulum (rer), often arranged in parallel series of cisternae, mitochondria and several Golgi complexes with vesicles. Smooth
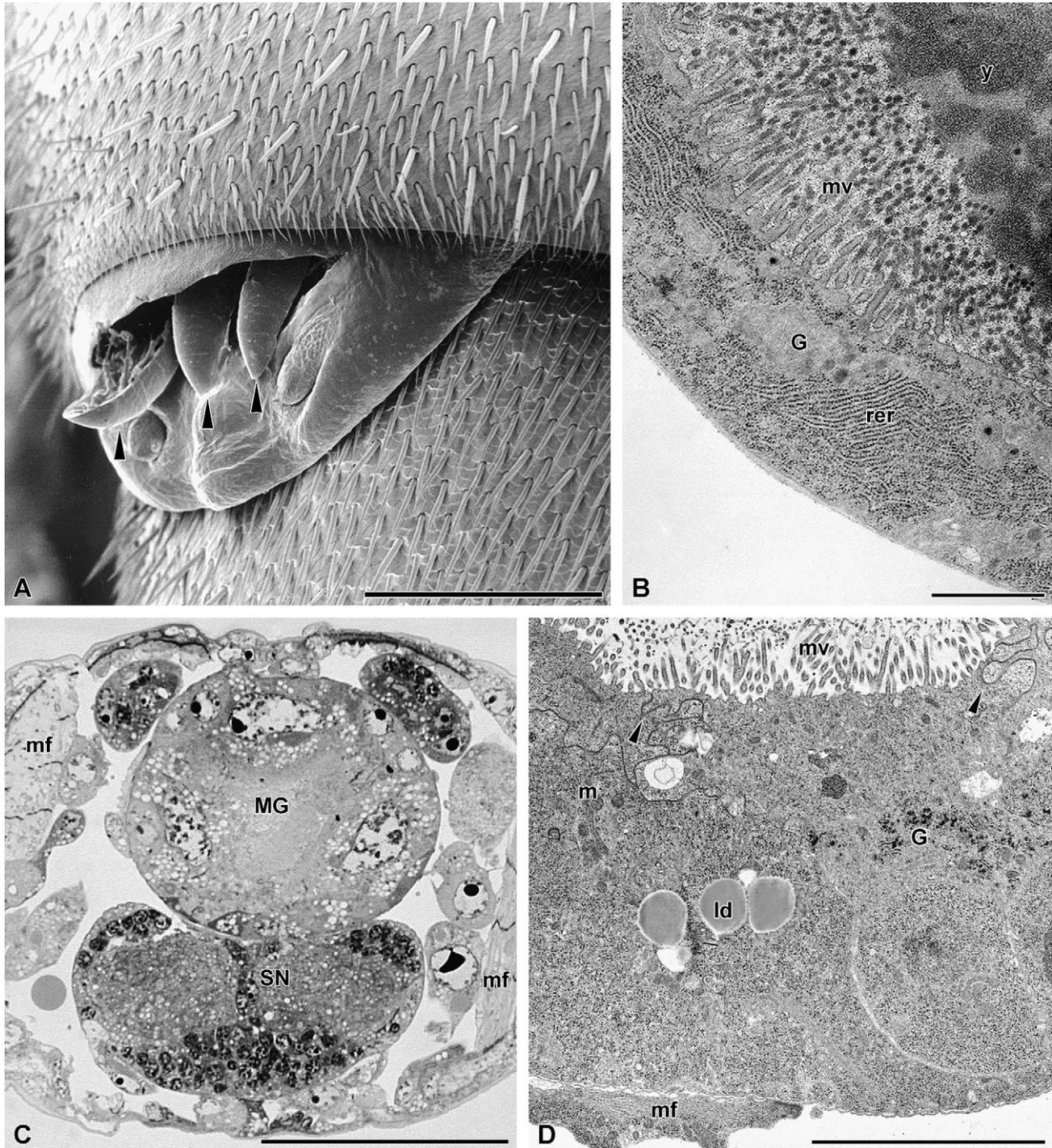

Fig. 2. (A) SEM micrograph of the last abdominal segments of the wasp *P. dominulus* with a neotenic female extruding with its "cephalothorax"; three triungulins are visible (arrowheads) just leaving the mother's body cavity through the brood opening. Scale bar 200 μm. (B) Cross sections through the midgut epithelium. Numerous long and sinuous microvilli (mv), rough endoplasmic reticulum (rer) with parallel arranged cisternae, several Golgi complexes (G) are present. Yolk masses (y) are evident in the gut lumen. Scale bar 1 μm. (C) Semithin section through the anterior body region of 2nd instar larva showing the midgut (MG) and the nervous system (SN) sectioned at a ganglion. Muscles fibers (mf) are visible at the peripheral region. Scale bar 40 μm. (D) Cross section through midgut cells of the 2nd instar larva showing microvilli (mv) provided with thin distal parts. In the cytoplasm lipid droplets (ld), few mitochondria (m), Golgi complexes (G) and beneath the epithelial cells muscle fibers (mf) are visible. In the apical region of epithelial cells sinuous continuous junctions (arrowheads) are present. Scale bar 5 μm.

septate junctions (continuous junctions) are visible between these cells. The luminal surface of the midgut epithelium displays numerous slender microvilli (about 1.3 μm) the basal region of which ($l = 0.5$ μm) is thicker (about 70 nm) while their thin distal ends extend sinuously into the gut lumen where large amounts of yolk are present. There is no evidence of a peritrophic matrix.

Two days after the 1st instar larva has penetrated into the host, it molts to the 2nd instar: an apodous unpigmented larva that is well visible through the host's cuticle due to two black spots corresponding to the eyes (Beani et al., 2005). At this stage the sexes are not yet distinguishable. A semithin section through the anterior body half shows that the midgut occupies the main part of the body, while the nervous system lies in the ventral region (Fig. 2C).

The midgut is a simple tube (approximately 40 μm in diameter). It consists of an epithelium laying on a thin basal lamina, surrounded by a few circular muscle fibers and some tracheae. The midgut cells are 9 μm high and are provided with a large central nucleus and numerous microvilli (1 μm long) disposed to form a brush border lining the gut lumen. These microvilli are similar to those seen in the previous instar showing a thin distal end. No peritrophic matrix is evident. The cytoplasm is rich in mitochondria, characterized by a dense matrix, free ribosomes, cisternae of rough endoplasmic reticulum, Golgi complexes and vesicles and a few large lipid droplets. Sinuous continuous junctions join the apical regions of neighboring cells (Fig. 2D).

At molting the 3rd instar larva, as previously described, retains the exuvium of the 2nd instar larva that, after a few hours, still adheres to the cuticle lining the epidermal cells (Fig. 3A and inset), whereas later it is separated from the body and appears as a thin layer (Fig. 3B). The midgut is

a cylindrical structure about 90 μm in diameter that occupies the main part of the body; some adipocytes are visible in the hemocoel. The small gonads begin to differentiate on both sides of the gut (Fig. 3A).

The midgut epithelium (Fig. 4A,B), which lies on a thin basal lamina, consists of a single layer of microvillated cells, 30 μm high, containing large polymorphic nuclei (Fig. 4C). At the base of almost orderly arrayed microvilli (about 3.25 μm), coated pits and dense secretory vesicles are recognizable. The apical cell region shows many mitochondria, several large lipid droplets and small secretory vesicles, closely connected with numerous Golgi complexes that contain a dense material (Figs. 4A—D and 5B). Large amounts of glycogen granules, lipid droplets and some lysosomes are also seen (Fig. 4D). In the basal region, plasma membrane infoldings give rise to narrow cytoplasmic compartments, where small mitochondria with a dense matrix are present (Fig. 5A). At this level we found regenerative cells provided with a rounded nucleus and an electron transparent cytoplasm rich in mitochondria (Fig. 5B).

If female, the 3rd instar larva undergoes the last molt without a pupal stage. At the early 4th instar, the cephalothorax, well recognizable by its constriction in the neck region, is unsclerotized until it extrudes from the host (Fig. 6A). At this stage the gonads progressively degenerate, so that the neotenic female, characterized by hemocoelic viviparity, lacks ovaries and oviducts. Oocytes can be seen free in the hemocoel and intermingled with numerous adipocytes which increase in number during this instar and progressively fill the hemocoel cavity (Fig. 6B,C).

Unextruded females still maintain an active midgut. Large midgut epithelial cells, laying on the basal lamina, show an elongated nucleus, numerous mitochondria (0.2×1.15 μm),
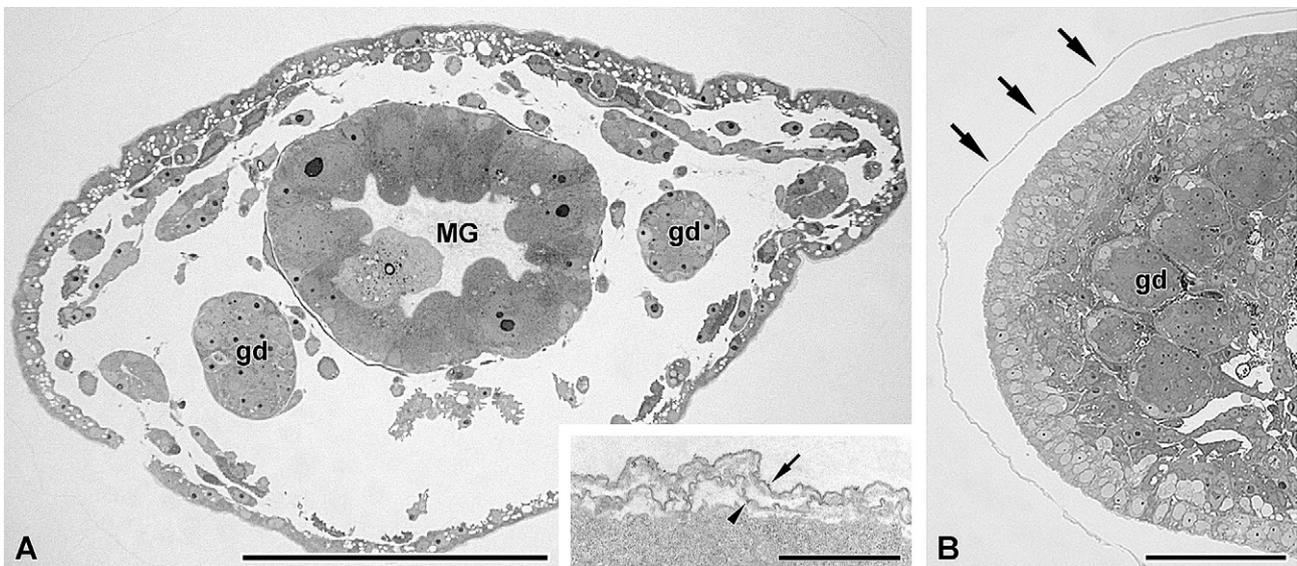

Fig. 3. (A) Semithin section of a 3rd instar larva, a few hours after molting; on both sides of the midgut (MG) the small gonads (gd) are visible. Scale bar 100 μm. The inset shows the exuvium of 2nd instar larva (arrow) still adherent to the cuticle lining of the epithelial cells (arrowhead). Scale bar 2 μm. (B) Semithin section of an older 3rd instar larva in which the exuvium of the previous instar surrounds at a distance its body (arrows). gd, gonads. Scale bar 25 μm.
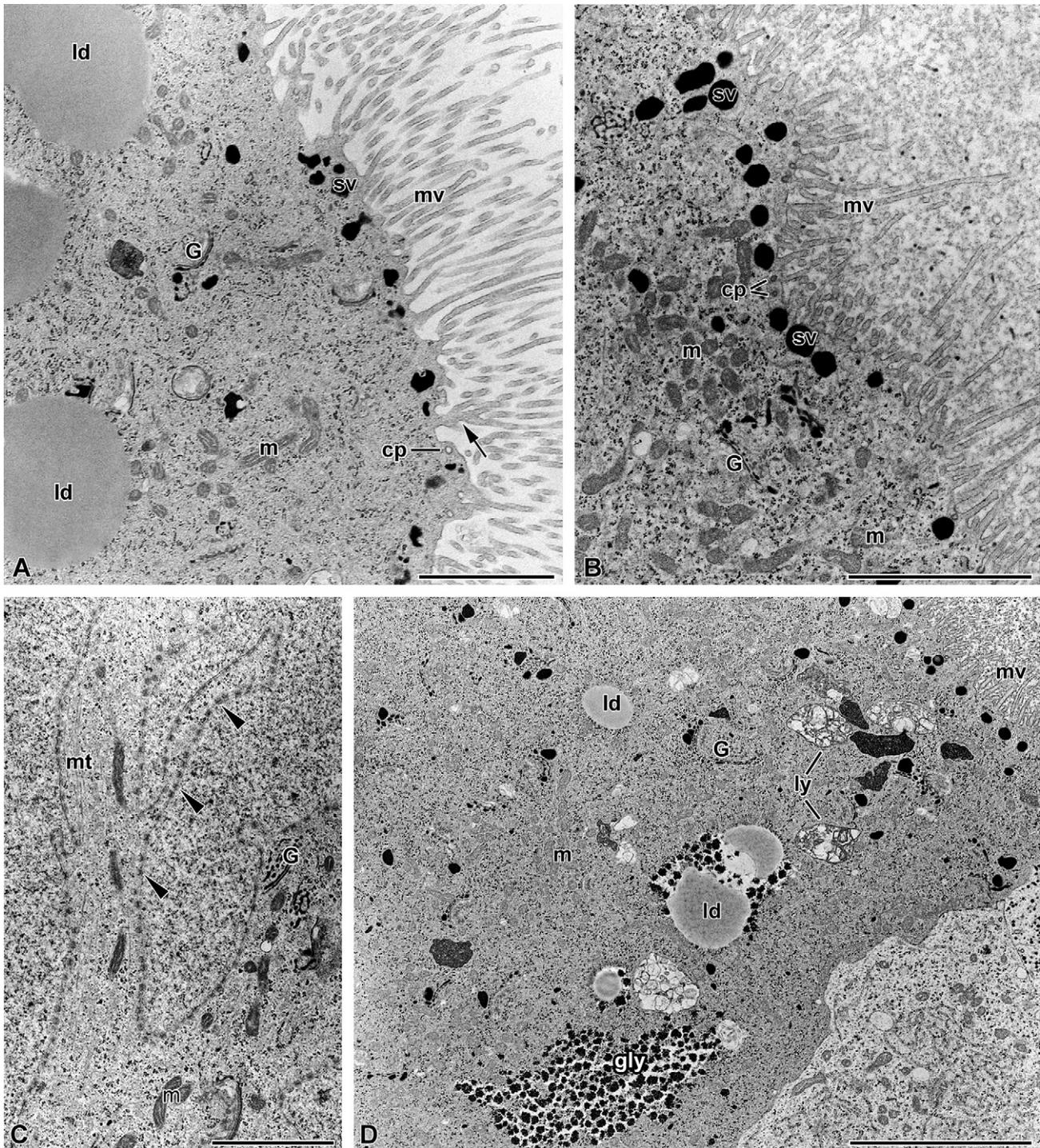
Fig. 4. (A,B) Cross sections through the apical region of epithelial cells of a 3rd instar larva. The microvilli (mv) are often clustered (arrow); at their base many coated pits (cp) and electron dense secretory vesicles (sv) are present. In the cytoplasm, mitochondria (m), lipid droplets (ld) and Golgi complexes (G) are recognizable. Scale bars 2 μm. (C) Electron micrograph of a 3rd instar larva showing a cell provided with a polymorphic nucleus rich in pores (arrowheads). G, Golgi complexes; m, mitochondria; mt, microtubules. Scale bar 2 μm. (D) Cross section through the midgut epithelial cells of a 3rd instar larva. In the cytoplasm a large amount of glycogen granules (gly), lipid droplets (ld) and some lysosome (ly) are evident. m, mitochondria; mv, microvilli; G, Golgi complexes. Scale bar 5 μm.

cisternae of endoplasmic reticulum, free ribosomes, Golgi complexes and lysosome-like bodies (Fig. 7A). Large amount of glycogen granules are stored apically beneath the microvilli (2.6 μm long) (Fig. 7B), where a few coated pits and small vesicles may be present (Fig. 7C) and many microtubules, mainly in the middle part of the cell (Fig. 7A).

3.3. Midgut ultrastructure of the extruded neotenic female

At the late 4th instar, the cephalothorax protrudes through the intersegmental membranes between the last tergites of the wasp. Soon after extrusion, the neotenic female may be
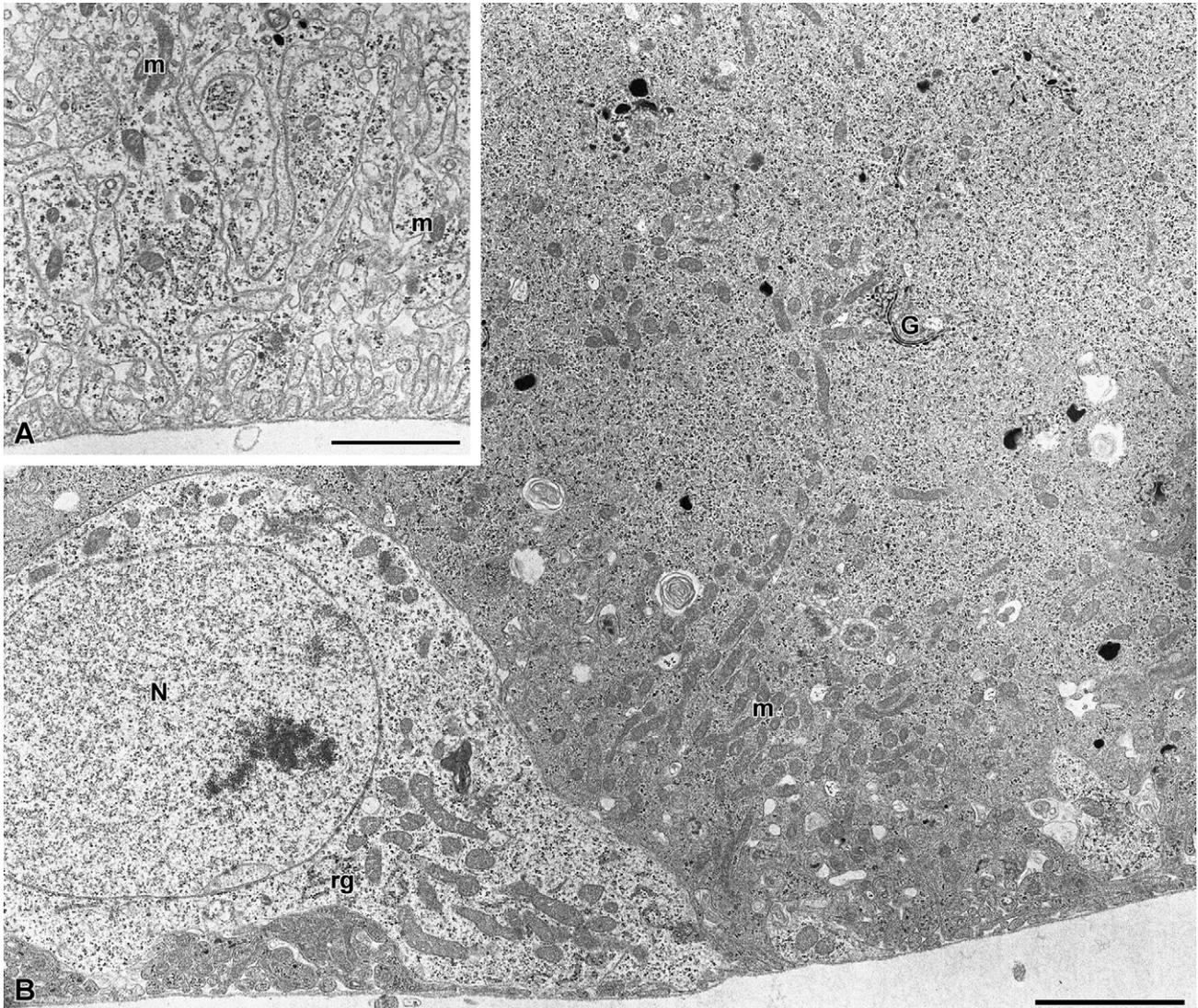
Fig. 5. (A) Cross section through the basal region of a 3rd instar larva midgut epithelial cells. Numerous infoldings of the basal plasma membrane which extend deep into the cytoplasm are present. m, mitochondria. (B) Basal region of the epithelial cells in which a regenerative cell (rg) is visible. N, nucleus; m, mitochondria; G, Golgi complexes. Scale bars 2 μm.

inseminated (Beani et al., 2005). A semithin section of a "young" fertilized female shows that the hemocoel is filled with embryos at different developmental stages, intermingled with adipocytes (Fig. 8A).

The midgut epithelium shows a dramatic progressive change in its ultrastructure in fertilized females of different ages. In the "young" females the microvilli are not arrayed in an orderly fashion and less numerous than before extrusion. Some epithelial cells have lost their microvilli. Large cell fragments in the midgut lumen might be remnants of degenerated cells (Fig. 8B). The cytoplasm is rich in mitochondria which are larger (0.65 × 1.65 μm) than in the previous instars (compare Fig. 7A with Fig. 8C); it also contains lamellar bodies, which likely represent autophagic structures (Fig. 8C). In the basal region, the plasma membranes form many deep infoldings, with which the large mitochondria are associated (Fig. 8B,C).

The midgut epithelium undergoes progressive degenerative changes in its ultrastructure, in overwintering *X. vesparum*

females developing embryos. The cells become narrow, only 5 μm high and protrude into the lumen; the cytoplasm is very reduced and contains many clustered mitochondria. The thin microvilli delimit a flattened lumen. The nuclei contain dense scattered masses of chromatin, resembling the nuclei of autophagic cell degeneration rather than apoptotic cell death (Sato et al., 2006); some of them are contained in large cytoplasm protrusions and some are free in the gut lumen (Fig. 9).

In samples of "old" females, releasing triungulins, the midgut epithelial cells form a thin layer, provided with some irregular microvilli extending into an empty lumen. The nuclei of these cells are no longer visible and the cytoplasm contains very few mitochondria and dense bodies of variable size. Sinuous septate junctional systems between cells (continuous junctions) are still well preserved. Beneath the thin epithelium, muscles cells are still visible, with longitudinal contractile fibers along the cytoplasm (Fig. 10A,B).
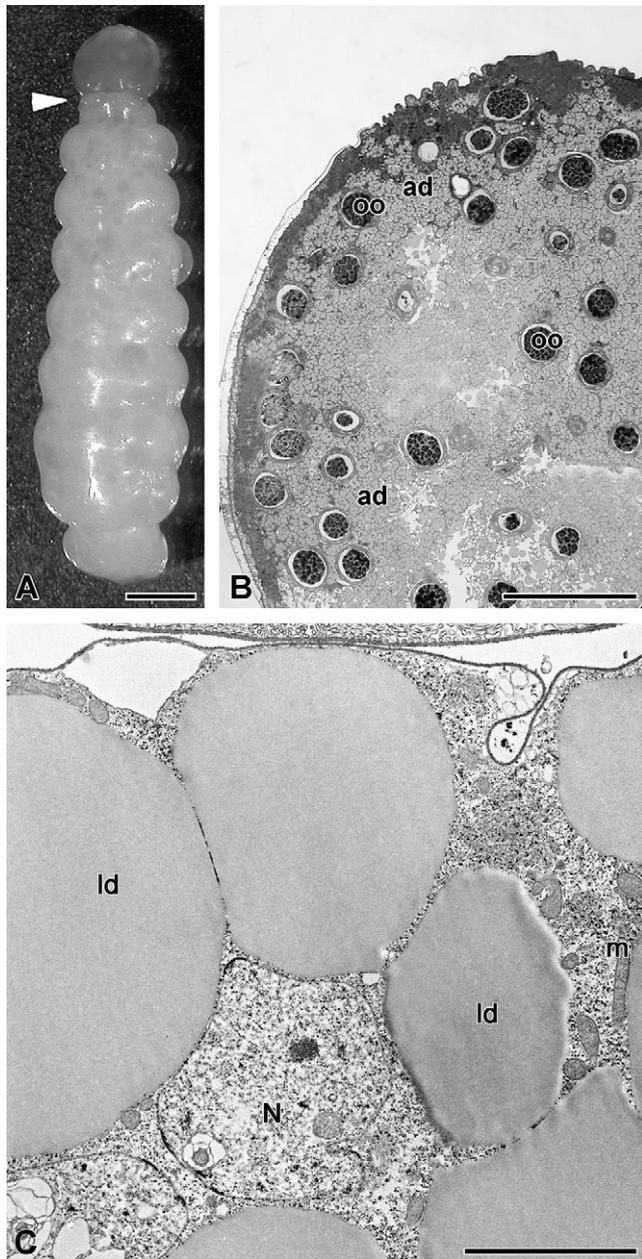
Fig. 6. (A) Ventral view of an unextruded 4th instar larva of *X. vesparum*. Note the constriction between the cephalothorax and the body region (arrowhead). Scale bar 1 mm. (B) Semithin cross section through an unextruded 4th instar larva showing the hemocoel filled with adipocytes (ad) intermingled with some oocytes (oo). Scale bar 250 μm. (C) Cross section through an adipocyte showing the nucleus (N) and lipid droplets (ld). m, mitochondria. Scale bar 4 μm.

3.4. Stable carbon isotopes analysis

In order to answer the question whether the parasite is able to uptake nutrients from the host after the extrusion of cephalothorax, we performed three experiments (Fig. 11; Exp. 1−3). In the first experiment (Exp. 1) we compared δ^{13}C values of "young" extruded *X. vesparum* females and of their hosts, which were always fed with beet sugar (X bs and Wp bs, respectively): the values were not different from expectation, i.e. similar values in

parasites and hosts ($\delta^{13}C_{X\ bs} = -25.06 \pm 0.61$ and $\delta^{13}C_{Wp\ bs} = -25.76 \pm 0.37‰$, respectively).

In a second experiment (Exp. 2), both parasitized and uninfected wasps (controls) were fed first with beet sugar and then with cane sugar. Three days after food turnover we measured δ^{13}C values and we found two opposite results: the δ^{13}C value of parasitized wasp (Wp 3d) as well as that of control wasp (Wc 3d) were visibly higher than those of Exp. 1 (-19.08 ± 0.32 and $-13.9 \pm 0.60‰$) due to the cane sugar taken by the wasps; on the contrary, δ^{13}C value from *X. vesparum* extruded female (X 3d) was almost unchanged ($-24.4 \pm 0.41‰$) compared to the value of Exp. 1, indicating that the parasite was unable to uptake cane sugar.

The third experiment (Exp. 3) was performed in a similar way to Exp. 2, but δ^{13}C values were analyzed 10 days after changing sugar. In this case the δ^{13}C values of parasitized and uninfected wasps (control) were similar to the results of the previous experiment with slightly higher δ^{13}C values (-16.2 ± 0.40 for Wp 10d and $-16.7 \pm 0.34‰$ for Wc 10d); on the contrary, the extruded *X. vesparum* female δ^{13}C value (X 10d) was unchanged ($-24.25 \pm 0.40‰$).

In order to answer the question whether the parasite is able to uptake nutrients from the host before its extrusion, we analyzed crypto-parasitized wasps (Fig. 11; Exp. 4 and 5). We compared δ^{13}C values from unextruded parasites as well as hosts fed only with cane sugar on the one hand (Exp. 4), and only with beet sugar on the other hand (Exp. 5). Exp. 4 showed similarly "heavy" δ^{13}C values both for the wasp (Wp cs = $-18.33 \pm 0.44‰$) and for the unextruded parasite (X cs unex = $-14.53 \pm 0.33‰$); on the contrary, Exp. 5 produced δ^{13}C values lower than Exp. 4 both for the host (Wp bs = $-25.68 \pm 0.45‰$) and for the unextruded parasite (X bs unex = $-25.45 \pm 0.47‰$).

4. Discussion

This study combines the analysis of midgut ultrastructure with the trend of sugar absorption in larval and neotenic *X. vesparum* females, in order to clarify the mechanisms of nutrient uptake in this host−parasite system. The midgut cells of this bizarre permanent endoparasite show changing ultrastructural patterns, according to both the roles that the midgut plays during larval development and to the absorption of nutrients from host hemolymph, which abruptly stops at the extrusion of female cephalothorax.

4.1. The 1st instar larva is not involved in host nutrient uptake

The 1st instar larva, after its exit from the ventral canal opening, is immediately able to search for and enter a larval host. Nevertheless, triungulins are resistant organisms, able to survive for several hours up to a few days out of the mother body. In spring an overwintered wasp, if parasitized by a fertilized *X. vesparum* female, could spread them on flowers, where they stay until a foraging wasp picks them up and transports them to a new nest (see Hughes et al., 2003, for infection
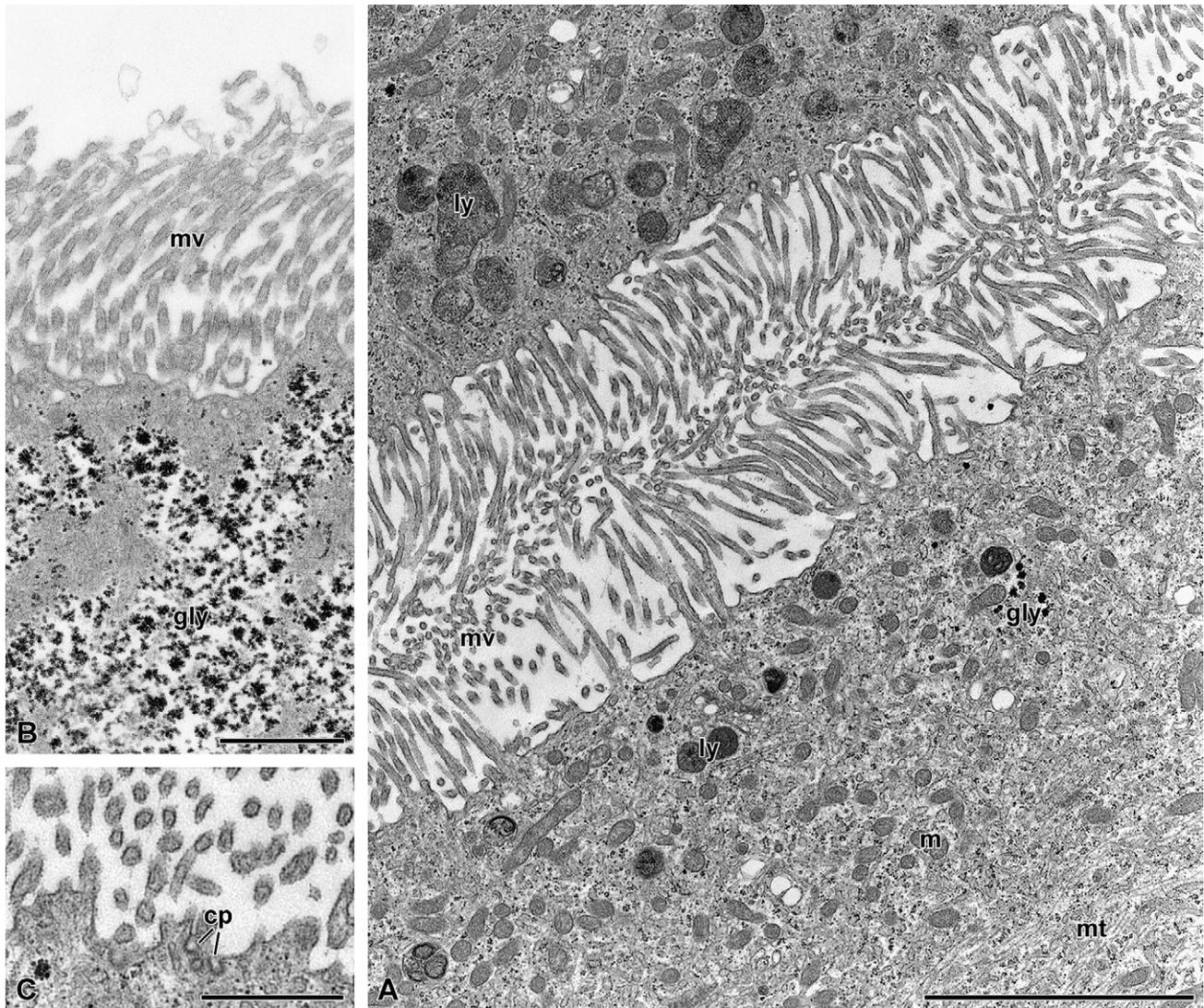
Fig. 7. (A) Cross section through the apical region of the midgut epithelium of unextruded 4th instar larva. Note the presence of many lysosome-like bodies (ly); many microtubules (mt) are visible in the middle part of the cell. m, mitochondria; gly, glycogen; mv, microvilli. Scale bar 4 μm. (B) Detail of the apical region of epithelial cells of unextruded 4th instar larva showing a big amount of glycogen (gly). mv, microvilli. Scale bar 1 μm. (C) Detail of the apical region showing coated pits (cp) beneath microvilli. Scale bar 1 μm.

modalities: phoretic transport and direct release close to combs). They show no strict environmental constraints: they are still active after one night in the fixative (personal observations). In terms of ''functional ecology of immature parasitoids'' and parasites (Brodeur and Boivin, 2004), this host-seeking larva is an efficient device to find and invade new hosts. They have functional legs and other morphological adaptations, such as long cuticular setae and tarsal pads on the first and second pair of legs, which help their displacement (Pohl, 2000) and adhesion to legs or abdomen of *Polistes* wasps (Hughes et al., 2003). A thick cuticle preserves them from dehydration.

As regards mouthparts, we have only observed a not yet analyzed secretion, perhaps from salivary glands, when they cross the wasp larval cuticle (Manfredini et al., in press). Ultrathin sections of the midgut of triungulins reveal the presence of large amounts of yolk in the gut lumen, which the parasite probably will use as a source of nutrients. Thus, this larva, during its short lifespan, might avoid the food uptake from the environment, as previously observed by Jeannel (1951) and also described in other insect species (Dow, 1986; Rost et al., 2005; Mori, 1983). The midgut seems not to be involved in secretory activities, even though epithelial cells are provided with organelles typical for secretions. The rough endoplasmic reticulum and Golgi complexes could rather synthesize digestive enzymes for the utilization of the yolk in the gut lumen.

4.2. Active synthesis by the midgut cells during the endoparasitic stages

In insects, it is well known that the midgut is the district specifically involved in the absorption and elaboration of nutrients (Wigglesworth, 1972; Martoja and Ballan-Dufrancais,
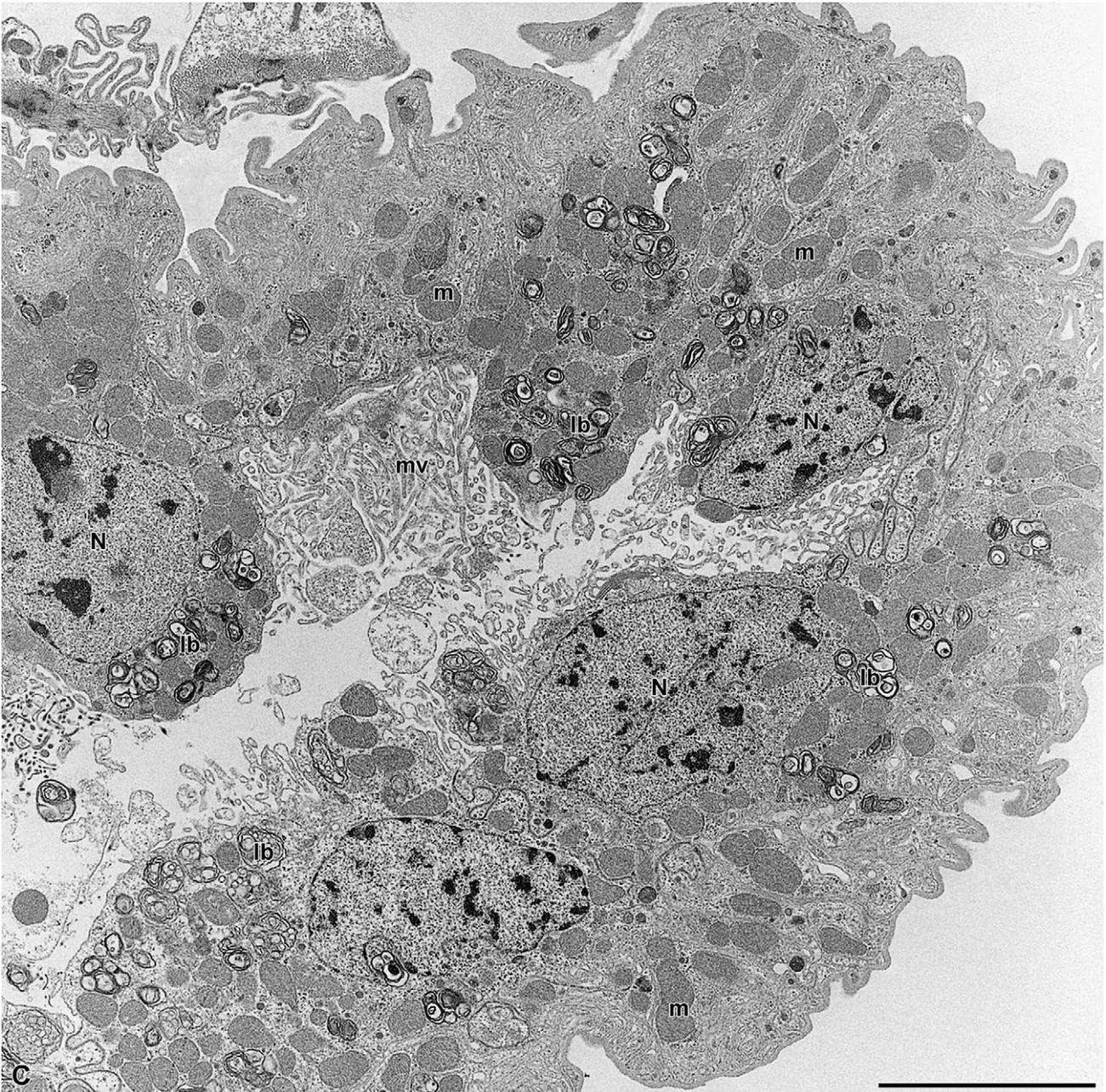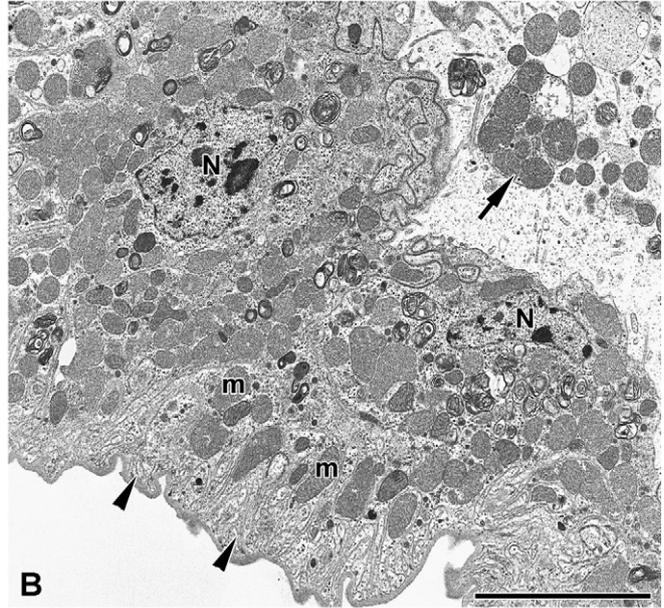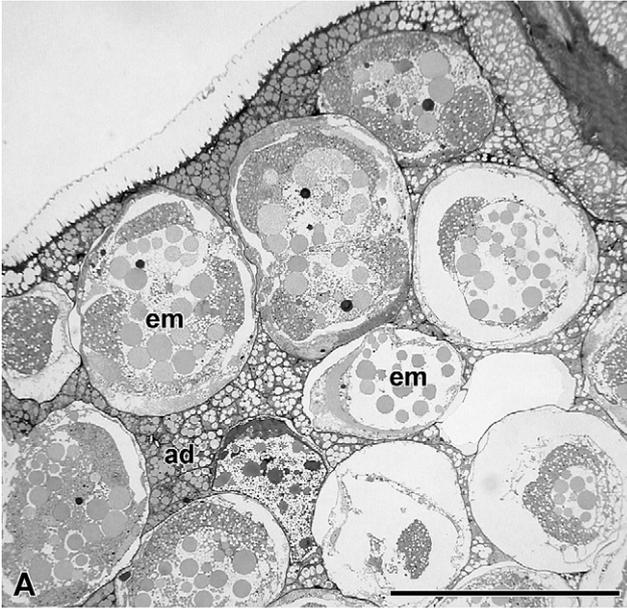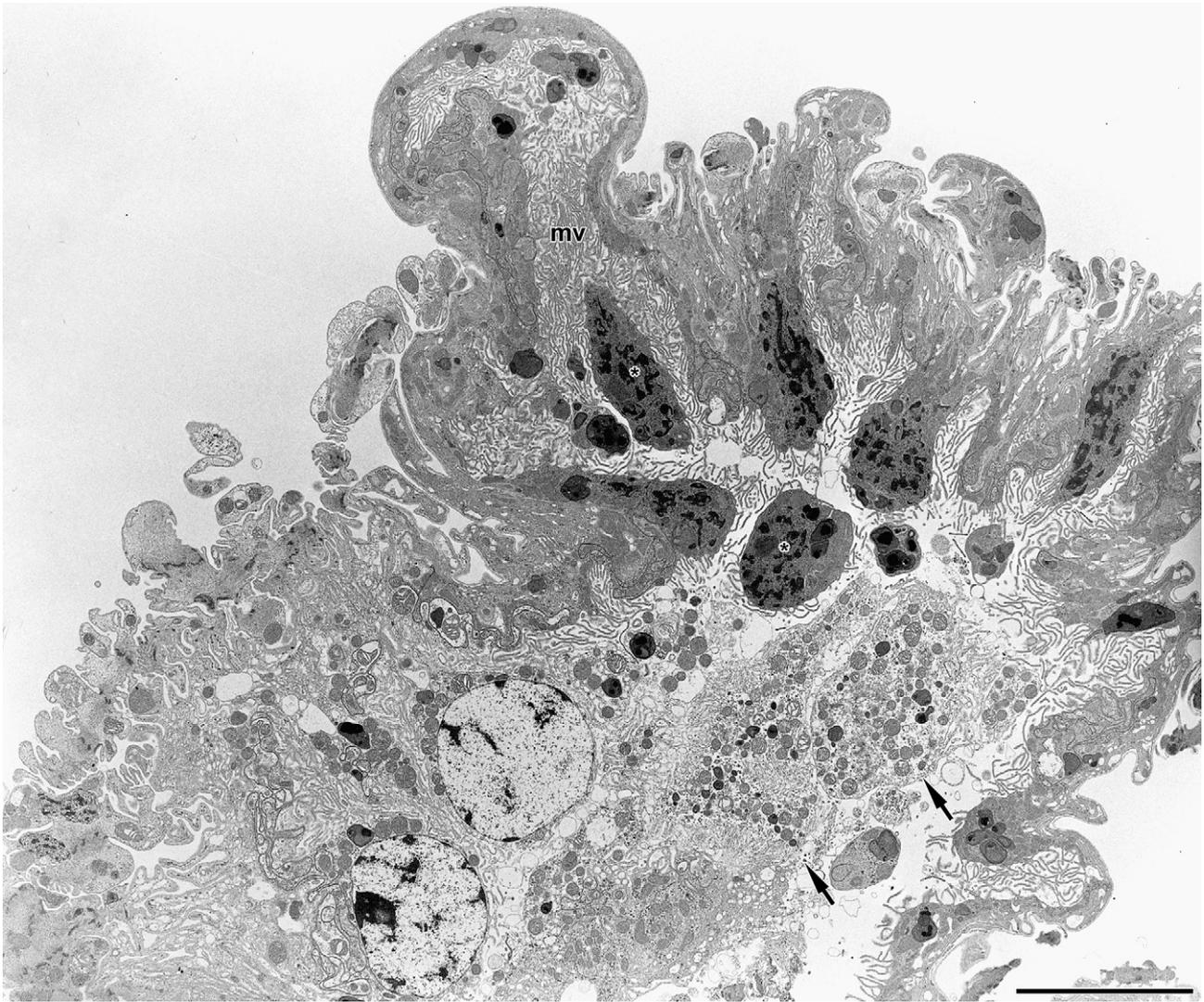
Fig. 9. Cross section through the midgut epithelium of a fertilized neotenic female extruded for 5 months. Some nuclei (asterisks) and cells fragments (arrows) are released into the gut lumen. mv, microvilli. Scale bar 10 μm.

1984; Dow, 1986; Lehane, 1998). The midgut epithelium of the 2nd and mainly of the 3rd instar larvae contains large nuclei, numerous mitochondria, free ribosomes, rough endoplasmic reticulum, Golgi complexes and vesicles. All ultrastructural features indicating an active secretion of digestive enzymes, synthesized in the rough endoplasmic reticulum, packaged in the Golgi complexes into secretory granules and released via exocytosis (Wood and Lehane, 1991; Billingsley and Lehane, 1996; Lehane, 1998). Numerous coated pits and vesicles suggest an endocytotic activity from the luminal content, and thus a nutrient uptake from the wasps hemocoelic fluid.

The storage of a great amount of glycogen in the unextruded female, allows us to hypothesize that the female starts accumulating nutrients into adipocytes, to be used later for the development of the embryos and for its own metabolism. The evident increase in number of adipocytes from 2nd to 3rd and 4th instars supports this hypothesis.

4.3. The progressive midgut modifications of the extruded neotenic female

The "young" extruded neotenic female has no longer a functional mouth through which it can take up nutrients from the host. The midgut shows a highly modified ultrastructural pattern: the epithelium is reduced and the organelles that are usually involved in secretion are less numerous, suggesting that the midgut is not involved in digestion and absorption

Fig. 8. (A) Semithin section of a neotenic female extruded for 2 months. Embryos (em) at different developmental stages, are visible intermingled with adipocytes (ad). Scale bar 100 μm. (B) Cross section through midgut epithelium of neotenic female extruded for 2 months. Only scattered microvilli are visible in the apical region; the plasma membrane, in the basal region, forms many infoldings (arrowheads) to which mitochondria (m) are strictly adherent. In the midgut lumen some cell fragments are visible (arrow). N, nucleus. C. Cross section through the midgut epithelium of neotenic female extruded for 2 months showing microvilli (mv) which are less numerous than in the previous instars; the cytoplasm exhibits many lamellar bodies (lb). N, nucleus; m, mitochondria. Scale bars 5 μm.
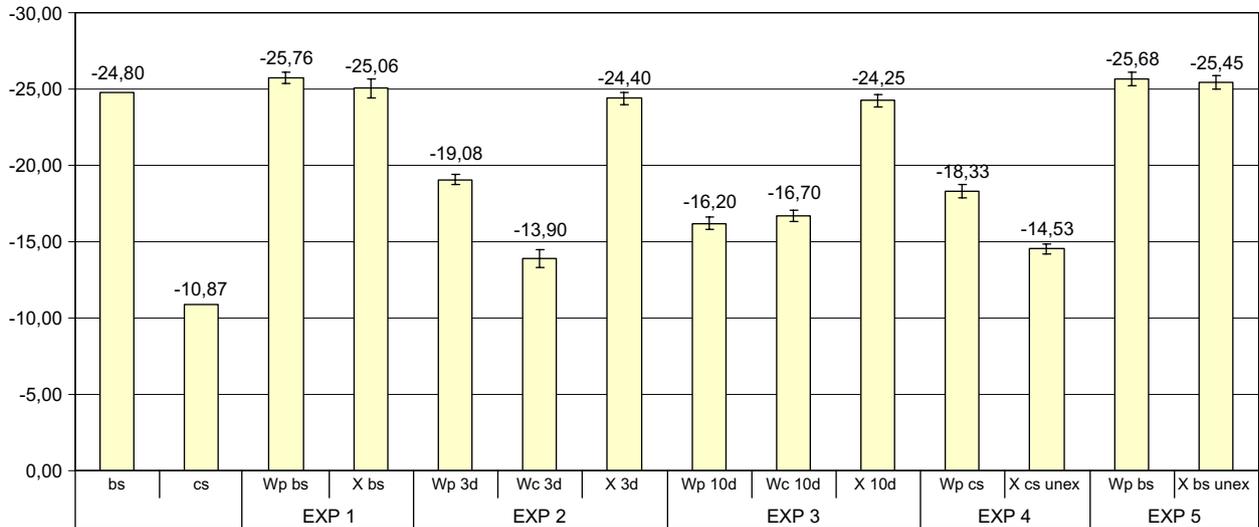
A

B
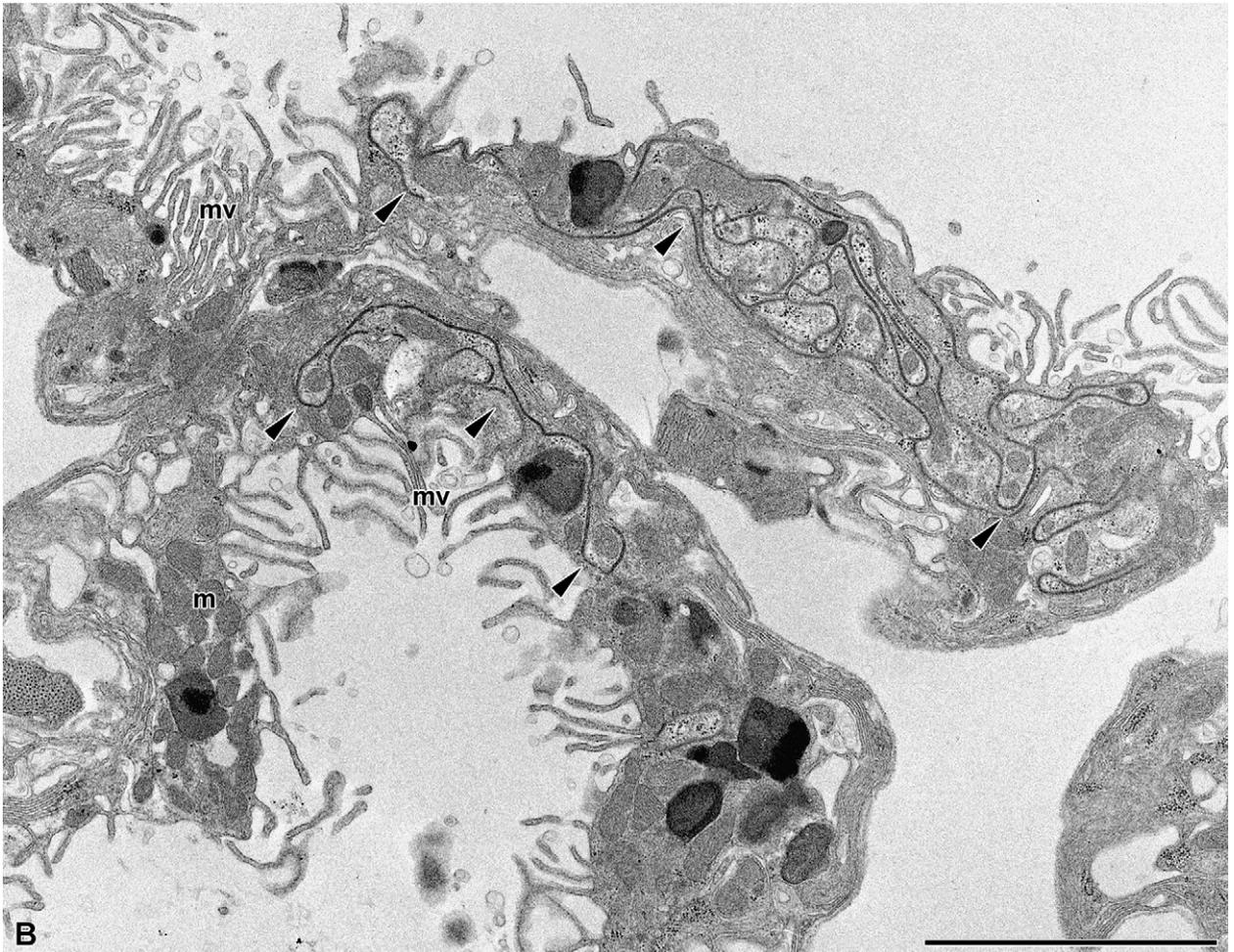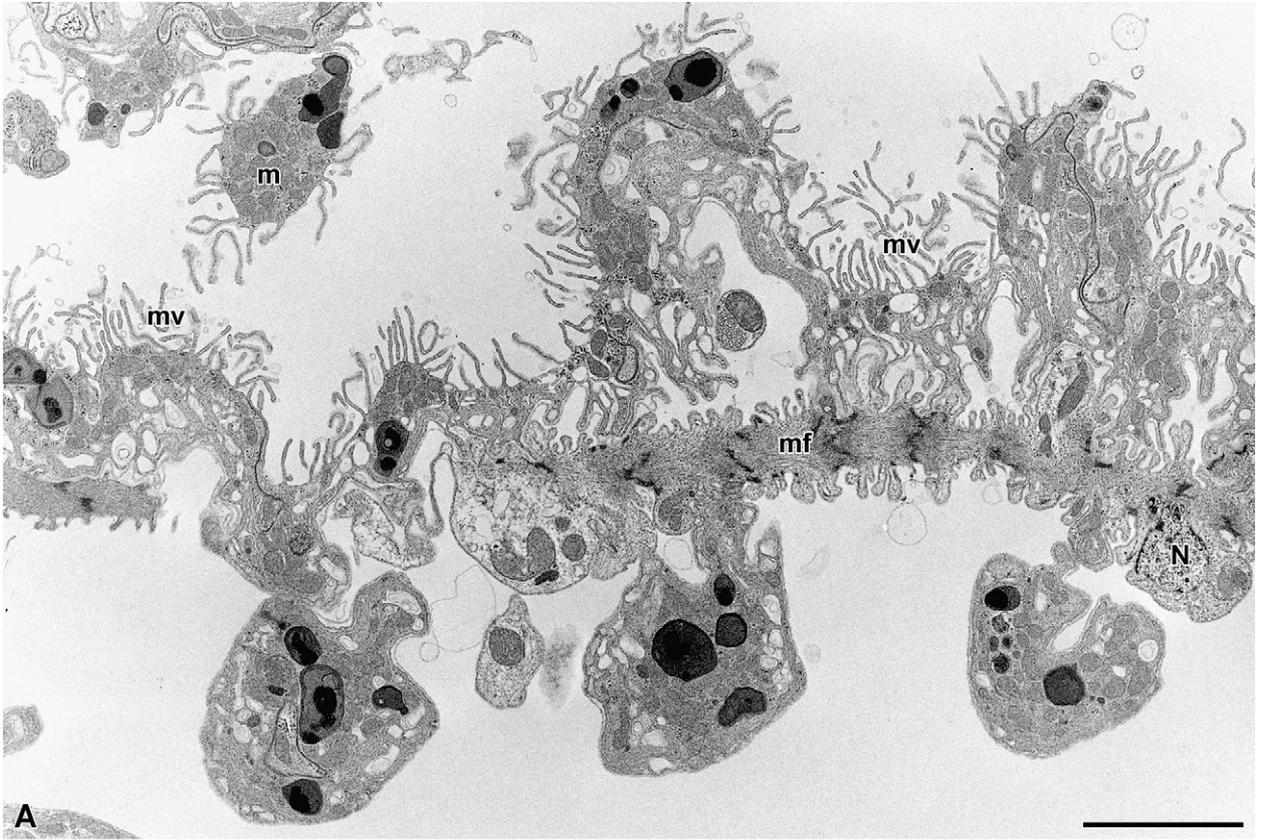
Fig. 11. $\delta^{13}C$ ($\pm$ SD) values obtained from experimental diet turnover (bs and cs indicate the $\delta^{13}C$ values of the beet sugar and of the cane sugar respectively). Exp. 1: Wp bs, parasitized wasps fed only with beet sugar; X bs, their extruded *X. vesparum* female parasites. Exp. 2: Wp 3d, parasitized wasps first fed with beet sugar and then with cane sugar for 3 days; X 3d, their extruded *X. vesparum* female parasites; Wc 3d, control wasps. Exp. 3: Wp 10d, parasitized wasps fed first with beet sugar and then with cane sugar for 10 days; X 10d, their extruded *X. vesparum* female parasites; Wc 10d, control wasps. Exp. 4: Wp cs, crypto-parasitized wasps fed only with cane sugar; X cs unex, their unextruded *X. vesparum* parasites. Exp. 5: Wp bs, crypto-parasitized wasps fed only with beet sugar; X bs unex, their unextruded *X. vesparum* parasites.

processes. At this stage, the midgut cells show basal membrane infoldings, which form a basolateral labyrinth intermingled with large mitochondria: a structural feature resembling the epithelium which regulates the transfer of solutes and water across the basal region and actively controls ion-exchanges (Berridge, 1970; Berridge and Oschman, 1972; Cioffi, 1984; Billingsley and Lehane, 1996; Lehane, 1998). If this structural analogy is correct, the midgut, before undergoing a complete degeneration, could play a role in the osmotic equilibrium of the hemocoelic fluids, in order to maintain appropriate conditions formerly for hemocoelic fertilization, and soon after for the embryonal development. This hypothesis is also supported by the temporal dynamic of these events: insemination may occur a few days after the extrusion of the cephalothorax (Beani et al., 2005), when Nassonow's glands, a possible source of sex-attractant pheromones, are fully active (Dallai et al., 2004); early development of embryos takes place before hibernation (see Fig. 8A).

In "overwintering" and mainly in "old" females, the midgut is degenerated and reduced to a thin epithelial layer, beneath which a consistent ribbon of muscles is present. At this stage, the nuclear release from the midgut cells is a critical step. This process is reminiscent of the autophagic cell degeneration and cell death, with the characteristic multilayered membranous bodies (Schweichel and Merker, 1973; Clarke, 1990). In this type of cell death, the nuclei are not pycnotic but contain still compacted and scattered masses of chromatin. Similar ultrastructural features have been observed in the resorption of the prothoracic gland at metamorphosis of *Manduca sexta* (Dai and Gilbert, 1997): the nucleus is first pushed apically, then it is included into a large drop of cytoplasm protruding into the gut lumen, and finally it is free in the lumen, with its dense masses of chromatin. However, in this involutive phase, the cell junction structures appear well preserved, as well as during the sea-urchin metamorphosis (Sato et al., 2006). This vestigial gut tube, filled with hemolymph which has lost its former function, might serve as a device to avoid the collapse of the inner body cavity.

4.4. The stable carbon isotopic analysis: a precise method to establish the trend of nutrient uptake

The isotopically labeled diet of *X. vesparum* larvae and neotenic females supports the ultrastructural evidence of the midgut as the chief site for both the synthesis of digestive enzymes and absorption of nutrients, until the extrusion of the cephalothorax. The stable carbon isotope compositions in wasps, which were fed with sugars derived from isotopically distinct sources, and in the female parasite, before and after its extrusion from the wasp, were directly influenced by the $\delta^{13}C$ of the assimilated food. Any dietary variation of the parasitized wasp from beet sugar, with very negative $\delta^{13}C$ values (-35 to $-21‰$; Deines, 1980) typical of C3 plants, to cane sugar with intermediate to low $\delta^{13}C$ values (-14 to $-10‰$; Ehleringer, 1991), characteristic of C4 plants, determined higher (less negative) $\delta^{13}C$ values.

Fig. 10. (A,B) Cross sections through the midgut epithelium of a neotenic female extruded for 1 year. Only a thin layer of cytoplasm provided with microvilli (mv) arranged in an unorderly fashion is visible. (B) A long continuous junction is present (arrowheads). mf, muscle fibers; m, mitochondria; N, nucleus. Scale bars 3 µm.

The comparison of δ^{13}C values between wasps and parasites likely reflected different extent of nutrient assumption. In particular, Exp. 2 and 3 clearly showed that the neotenic female was unable to assimilate the cane sugar with which the wasp was later fed after the extrusion of its cephalothorax, but it retained the same δ^{13}C typical of the beet sugar. In Exp. 4 and 5, the unextruded *X. vesparum* female was able to take up cane/beet sugar used as feeding source for the wasp. The δ^{13}C values of the wasps and parasites fed with beet sugar are similar and even more negative than the δ^{13}C values of the assimilated food, whereas none of the analyzed specimens has shown to approach cane sugar δ^{13}C values. Both these features contrast with the ''metabolic effects'' expected to produce animal δ^{13}C values isotope slightly higher ($\sim 1\permil$) than dietary source (''trophic shift''; De Niro and Epstein, 1978). Further investigation will be needed to characterize the carbon isotope fractionation during storage and assimilation of lipids (adipocytes) by the unextruded parasite. It is noteworthy that the results provided by the carbon isotope method agree with those of Strambi and Strambi (1973b), based on labeled amino acids: after 6 h, the silver grains were more abundant in the fat tissue of unextruded *X. vesparum* females, but not in ''overwintering'' females. Moreover, around 15 days post-eclosion, the level of hemolymph protein in crypto-parasitized wasps was significantly lower than in the healthy ones, suggesting an ''intense metabolic activity'' of the parasite, which at the late 4th instar undergoes a great increase in size (Strambi et al., 1982). The relatively low cost of parasitism for immature wasps is equally explainable in terms of a limited and ''slow growth'' during the host's critical developmental stages (Hughes and Kathirithamby, 2005).

4.5. The extruded neotenic female is unable to uptake nutrients from the host

Concerning the previous hypotheses of nutrient uptake through the cuticle of the parasite, recently revisited in a different species and family of Strepsiptera (Kathirithamby, 2000), our study seems to diverge completely. Any parenteral nutrition is here unlikely, because the cuticle of the *X. vesparum* female (covered by spines, not microvilli) is surrounded by two previous larval cuticular exuvia (see Beani et al., 2005); it is hard to think that nutrients can pass through these acellular layers, which form continuous rather than perforated envelopes. The absorption through the tegument as described in tapeworms is performed by an active and peculiar cellular syncytium that acts as an interface of the worm with the luminal contents of the host intestine (Holy and Oaks, 1986; Coil, 1991). Noticeably, *Stichotrema dallatorreanum*, the strepsipteran equipped by *apron*, ''has no fat body'' (Kathirithamby, 2000), whereas the neotenic *X. vesparum* female, after its extrusion from the host, has already stored in the adipocytes the amount of reserves needed for its metabolism and for the survival of its embryos. In this sense, the extruded female, after mating and egg fertilization, has finished its main role; it becomes only a protective and suitable container rich of adipocytes and tracheae for thousands of new larvae that will

progressively escape from the ventral brood opening. In line with a sustainable exploitation of the host, wasps infected by *X. vesparum* females spend their inactive but long life out of the colony, without any nutritional depletion (Hughes and Kathirithamby, 2005; Strambi et al., 1982) by the parasite after its extrusion.

Acknowledgments

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