POST- EMBRYONIC DEVELOPMENT OF OVARIOLE PEDICELS IN *Triatoma infestans* (KLUGH 1834). A MORPHOMETRICAL APPROACH.

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ABSTRACT
Triatoma infestans (Klug 1834) is a reduvideae hemiptera, one of the main vectors of *Trypanosoma cruzi*, the Chagas decease agent in American Continent. The post embryo pedicel development is studied. The pedicel is a part of the ovarioles not completely described in the literature in particular for embryo and post embryo development of telotrophic ovaries in hemiptera heteroptera insects. The histological and ultra-structure modifications of such ovariole component, already present at hatching time, have been followed for each nymph instar. Pedicel length growth have been measured in all instars. The secretory activity of pedicel epithelium has been morphometrically analyzed, from the third instar up to the fifth. An assessment was also made with just molted adult females ovarioles. The present work revealed that pedicel differentiation and cell activity start in the third nymph instar. The secretion product that fills the pedicel lumen and accumulates step wisely in the oviduct is different from the one found at the adult. Some probable pedicel functions are considered.

Key Words: Female gonad, pedicel, differentiation, growth, Hemiptera Reduvideae

RESUMEN
El hemíptero – reduvideo *Triatoma infestans* (Klug 1834) es uno de los principales vectores de *Tripanosoma cruzi* protozoo responsable de la enfermedad de Chagas en el continente americano. Se ha estudiado en esta especie el desarrollo post-embrionario del pedicelo. El pedicelo es una parte de la ovariola no completamente descripta en la literatura, en particular para los estadios de desarrollo embrionario y post embrionario en ovarios telotróficos para Hemíptera – Heteróptera. Las modificaciones histológicas y ultraestructurales de esta región de la ovariola, presente ya desde la eclosión, han sido analizadas en cada uno de los estadios ninfales. La longitud y crecimiento fue medido en cada estadio. La actividad secretoria del epitelio pedicelar fue analizada morfometricamente a partir de las ninfas del tercer estadio hasta el quinto. El producto de secreción que llena la luz del pedicelo y se acumula paso a paso en el oviducto, es diferente de aquella encontrada en estado adulto. Sobre la base de los datos obtenidos, las probables funciones del pedicelo son consideradas.

INTRODUCTION

Development and differentiation of telotrophic ovaries have a rather scarce bibliography. Important works as those presented by [1,5], pointed out adult gonads developmental details although their main reference are devoted with the relationship between nurse cells and oocytes, their organization, differentiation and maturation in particular the last one.

In *Triatoma infestans*, (Hemiptera Reduviidae), we have performed studies dealing with the ovaries and female gametes [6,8], spotting at the present our interest in embryo and post-embryo development of the pedicel of the ovariole, considered the unit of the female gonad.

The ovary is made up of seven ovarioles. In the adult stage each one is composed by four regions. The distal one opposite to the filament, is the pedicel, as stalk like stem that make connections between each ovariole with the lateral oviduct. Few studies have been presented describing this ovariole zone [9], referred to the pedicel in *Triatoma infestans* adult ovary as a short part composed by simple epithelium and a thick basal membrane. The presence of the so-called "annular gland" is also referred too. Very few is known in dtail about this ovariole region in Hemiptera. References about development and differentiation of the pedicel after the post embryo instars, are also scarce.

The present work evaluates the changes in pedicel from just hatched first instar nymphs up to fifth instar nymph ready to molt to adult. This work shows the structural cell characteristics correlated with its functional state and their participation with the whole organized organ. A morphometrical study of pedicel length and lumen surface variation throughout metamorphosis is also presented.

MATERIAL AND METHODS

Triatoma infestans from ova were raised in the laboratory, maintained at 27 ºC and fed on a regular basis.

Before dissecting the nymphs were narcotized for a few seconds with chloroform. An in situ fixation was performed with freshly prepared Karnowsky's fixative. The ovarioles were fixed at approximately 8° C for no less than 30 minutes. For the 4^{th} and 5^{th} instars dissection was performed both with just molted specimens, and ready to molt to the next stage.

After fixation the samples were washed twice in cacodilate buffer 0,1 M pH 7,4. Post fixation was performed with a 1% osmium tetra-oxide solution in the same buffer for the next 2 hours at 4ºC. When post-fixation was finished the specimens were dehydrated with an increasing ethanol series and two passages through propylene oxide.

Samples were embedded in Poly Bed – Araldite resin according to [10].

Sections for light microscopy 2μm thick, were cut with an LKB microtome, stained with azure II – methylene blue and mounted with the same resin used for embedding. Sections for electron microscopy were also obtained with LKB microtome and contrasted with acetate uranyl and lead citrate according to the process described by [11]. Photographs were taken with a Zeiss 109 TEM (ultra-structure) or a Zeiss Photomicroscope (light cytology) using Kodak T-Max 100 as negative recording material, processed with D19b developer.

Morphometry

Pedicel length was measured on at least 35 specimens for each instar, under a Zeiss Photo-microscope with a Kpl 12,5x micrometric eyepiece using whole fixed ovaries, stained with Congo red, placed on depression slides.

The morphometrical study was performed on 50 scanned photomicrographs –either optical or electronic– of trans-section ovaries of each nymph instars; have been analyzed using Image Pro Plus program (Media Cybernetics). The following parameters were measured:

1. ¨The surface occupied by the pedicel lumen,
2. The surface occupied by discharged vesicles in the pedicel lumen.

RESULTS

Similar characteristics were shown by nymph instars I and II ovarioles to those presented by the embryo at the hatching (Fig. 1). It is difficult to find any significant difference other than size between both instars. Observations were made on nymphs dissected at different times from twenty-four hours up to ten days after the molt.

Three well distinct regions compose the ovarioles within this period of post embryo development: the filament, the body and the pedicel (Fig. 2 and Fig. 3).

The last mentioned compared with the other two has a surface and volume quite similar. Such pedicel, the region nearest to the caudal ovariole end is formed by a rather compact cell complex not yet fully organized. Each cell shows large nucleus inside them conspicuous nucleoli are presented. No evidence of a high cell activity is observed under the TEM, there are few mitochondria; rough endoplasmic reticule and Golgi are scarce.

As the third nymph instar is analyzed important changes regarding size and structural form as well as cell activity is easy to find. The pedicel length increases

in this instar compare with the two previous, as expressed on graph (Fig. 4).

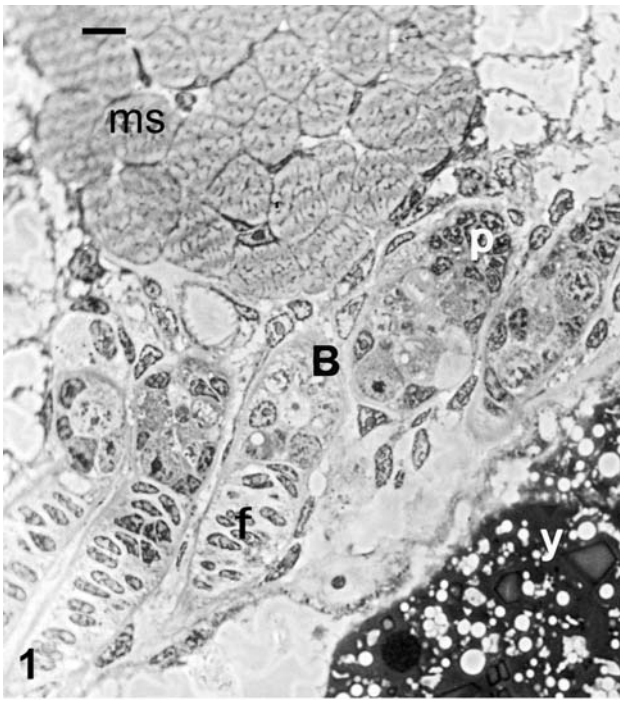


Fig. 1. Longitudinal section from a fourteen (14) days embryo, getting ready to hatch. Five ovarioles can be observed which regions are: f→ filament; B→ body; p→ pedicel. Toward a side of the ovary a muscle mass is present → ms; the opposite side is occupied by the remaining vitellus mass (yolk) → y, still together to the embryo. (Bar = 10 μm)

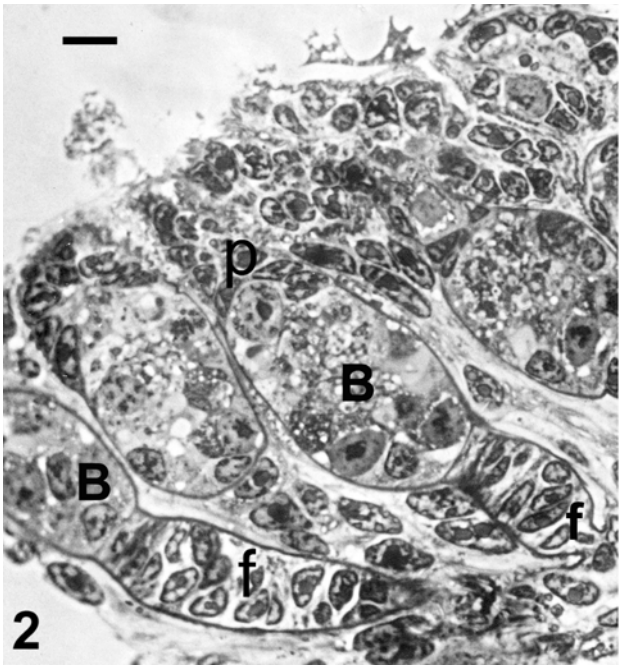


Fig. 2. First instar nymph ovary section; two ovarioles can be observed with their regions: f→ filament; B→ body; p→ pedicel. (Bar = 10 μm)

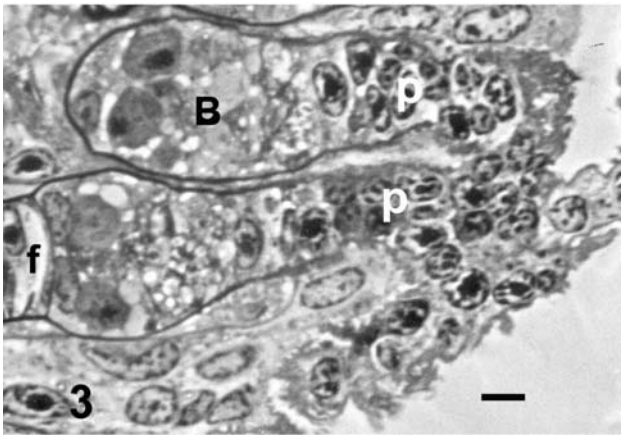


Fig. 3. Second instar nymph ovary section where the zones visualized are: B→ body; p→ pedicel, f.→ filament (Bar = 10 μm)

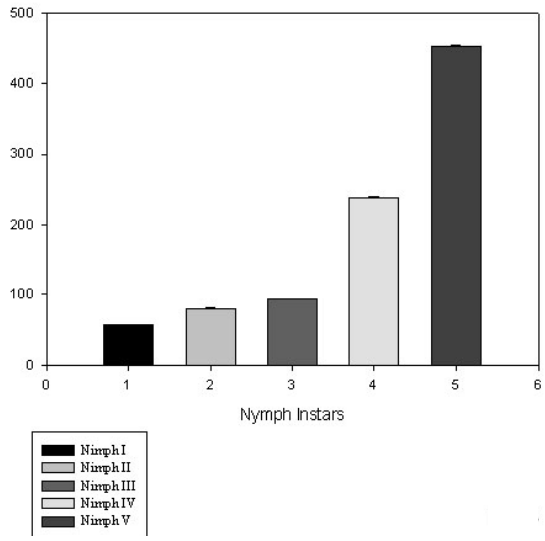


Fig. 4. Pedicel total length changes during nymph instars first to fifth. Data are expressed as Mean ± s. e. m. in μm

Now the epithelium is formed by cylinder like cells, their nuclei are conspicuous as are the nucleoli (fig 5). The pedicel proximal zone shows in the lumen vesicles contents, particularly surrounding the tube wall. Following the duct in its length toward its junction with the lateral oviduct no vesicles are observed, instead fibrillar structures, not well defined under the light microscope due to their size, can be seen in the lateral oviduct.

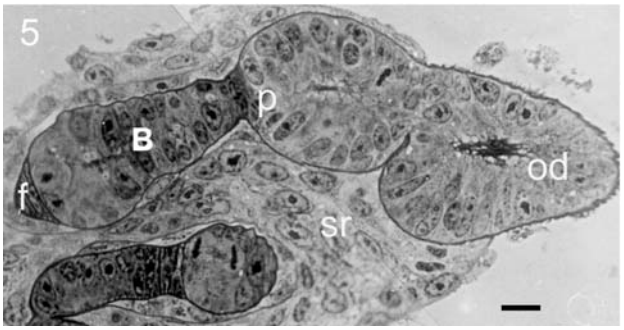


Fig. 5. Third instar nymph ovary section. Two ovarioles show their regions: f→ filament; B→ body; p→ pedicel; od→ oviduct; sr → stroma. (Bar = 10 μm)

The epithelium cells submicroscopical structure shows large nuclei, conspicuous nucleoli, rough endoplasmic reticule (R.E.R.), Golgi and mitochondria localized at a cytoplasm cell side (Fig 6a, 6b). Septata type cell junctions are present, cytoplasmic bridges between cells are found; their outlook is similar to those found in the upper parts of the ovariole, although in fewer number. At this part ovariole portion the cells convey an exocytic functional stage, at pedicel apical zone it is rather more evident (Fig 6b).

The studies of fourth instar nymphs allow us to assert that in such period the pedicel development and differentiation augment. The length increase is notorious and is maintained throughout this instar up to the fifth. The proximal region can now be divided in two areas: a little one proximal to the germarium, which has the appearance of a collar, its structure is somewhat compact, and another composing the main pedicel body which has a typical duct structure, its cavity is surrounded by column cells.

The micrographs presented came from a just molted fourth instar nymph (Figs. 7 and 8), and from samples near to molt to the fifth instar (Fig. 9) The histology, cell type and cell order are similar to the one found in third instar nymphs.. Intermingled with the column cells, dense cytoplasm cells establish themselves (Figs. 9 and 10c).

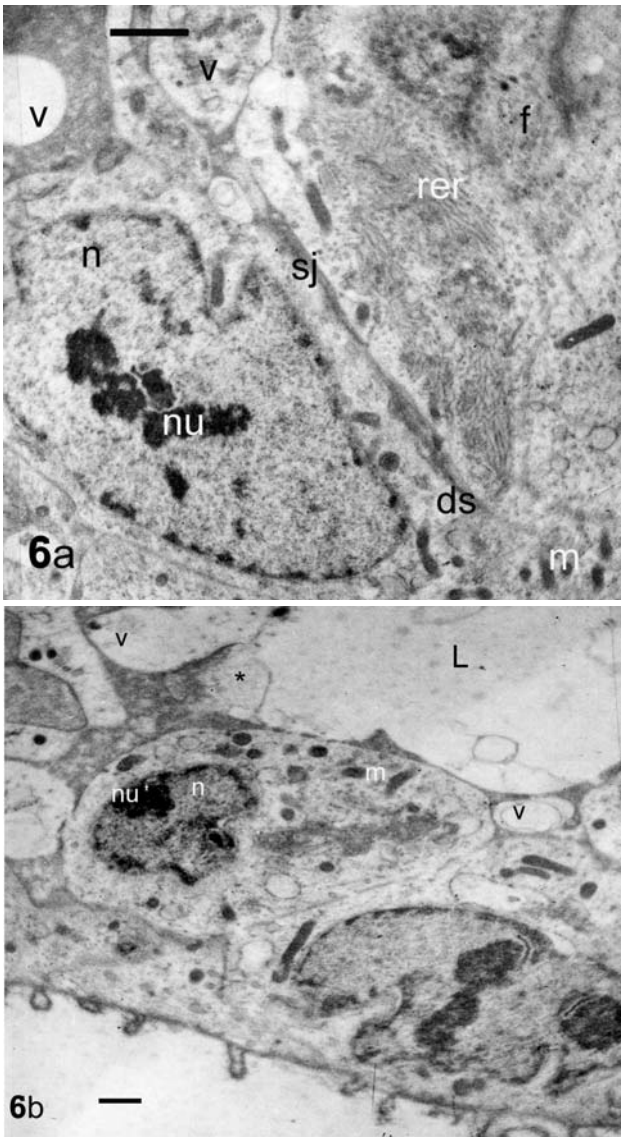


Fig. 6. Thin section TEM micrograph showing third instar nymph pedicel epithelium ultastructure.

6a. Epithelial cell. n→ nucleus; nu→ nucleole; rer→ rough endoplasmic reticule; sj→ septata junctions; ds→ desmosomes; v → vacuole. (Bar = 1 μm)

6b. Pedicel epithelial cells showing a secretion process. v→ free vesicles in the L→ lumen, and some near to be liberated; n→ nucleus, nu→ nucleolus, m→ mitochondria. (Bar = 1 μm)

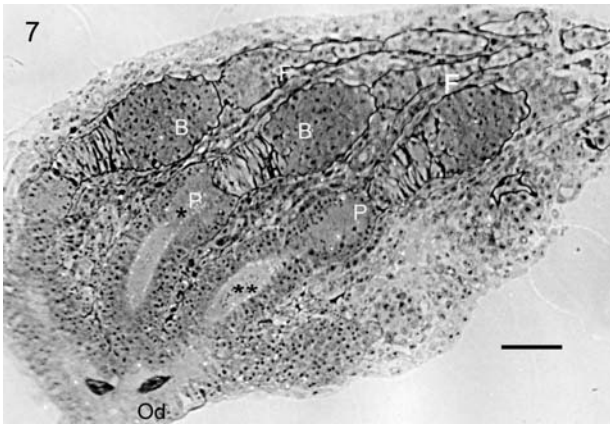


Fig. 7. Initial (just molted) fourth instar nymph para-sagital ovary section. Three ovarioles can be distinguished. f→ filament, B→ body, P→ pedicel. The proximal pedicel zone shows secretion vesicles in the lumen (*). In the distal pedicel zone, the lumen is occupied by the secreted product (**). The junction of two pedicels with the lateral oviduct→ (Od) can be observed, two lumen are plenty of the secreted product. (Bar = 50 μm)

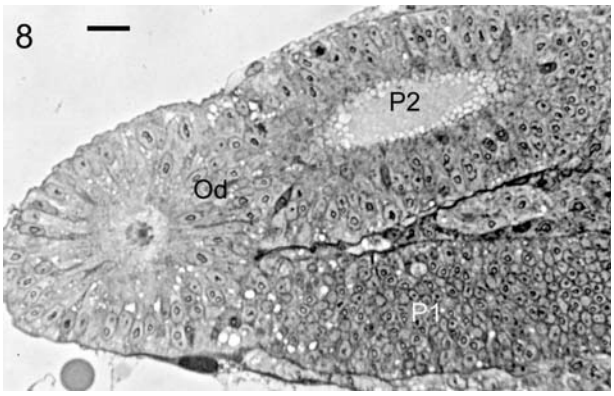


Fig. 8. Fourth instar nymph ovary section showing two ovarioles sectioned at distinct levels; one at the wall level (P_1), and other in the lumen (P_2) joining the lateral oviduct→ Od; (Bar = 10 μm)

Submicroscopical changes in the cell structure are observed, the mitochondria are scattered in the cytoplasm, large nuclei with multiple nucleoli, RNA is found circling the DNA; ER and Golgi apparatus are conspicuous. (Figs. 10a, 10b, 10c). Secretion activity shows an important increase too. The proximal pedicel zone up to the middle time of this instar, presents at the lumen periphery secretion vesicles, but as time goes by, the total lumen is filled, chiefly as the instar is reaching the molting period. On the other hand at the distal pedicel, joining the lateral oviduct link, the lumen shows scarce vesicle number, instead a fibrillar product denser than in previous instar is set up (Fig. 9).

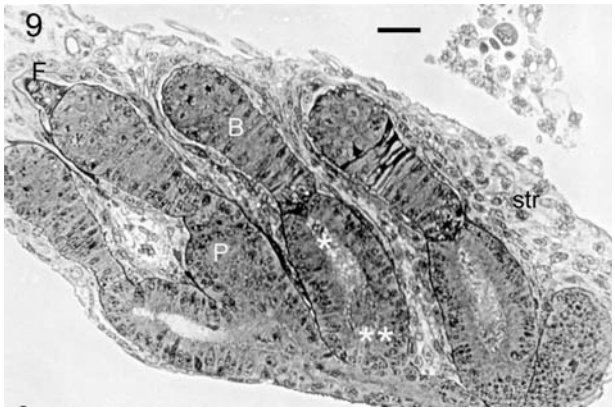


Fig. 9. Fourth instar nymph ovary section at the end of the period, near to molt to the fifth instar. The proximal pedicel region is shown where the vesicles secretions now occupied all the lumen (*). In the caudal zone vesicles and products can be observed, the last one in larger quantity occupies the lumen (**); F→ filament; B→ body; P→ pedicel; str →stroma. (Bar = 20 μm)

Fifth instar molts show from their beginning more noticeable anatomical and morphological changes. Pedicel length increase reaches its climax. Fifteen days after the molt the proximal pedicel zone presents a thicker column cell epithelium. The number of dense cytoplasm cells is larger than those found in 4th instar, some of them with such a huge size that called our attention (Figs. 11 to 13). The ovariole stroma seems to be activated at this instar too, a secreted product now found among their cells seams to all appearances to flow through the basement membrane into the pedicel lumen. It appears to be running via the intercellular spaces giving rise to structures that we might name "lacunae" (Fig. 11).

Submicroscopic structure shows that ER, Golgi apparatus and microtubules are now very conspicuous. Predominant cell junctions are still of the septata type, but a more frequent number of gap junctions and desmosomes, both between cells and bridges are present (Fig. 12 - 14a - 14b).

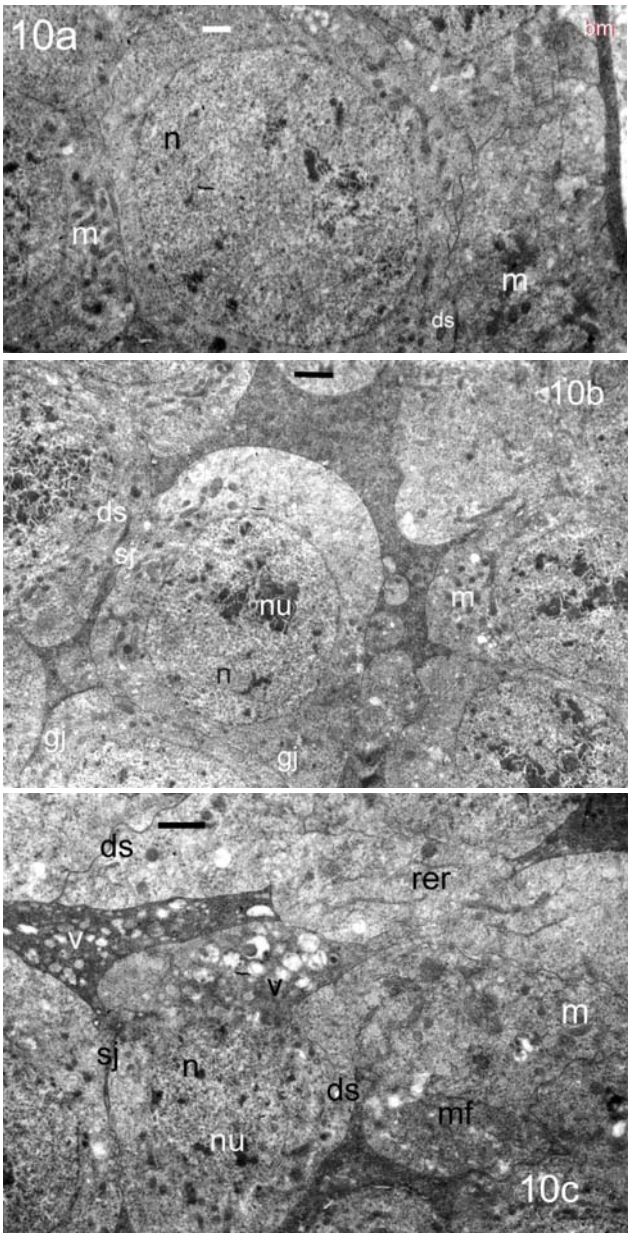


Fig. 10. TEM pedicel thin section micrograph. Two zones of the epithelium are shown:

10a. The adjacent to the basal membrane cells, n→ nucleus with multi-nucleoli, m→ mitochondria scattered all over the cytoplasm.

10b and 10c At the medial epithelium zone toward the lumen, intercalated with typical cells, dense vacuolated cells are present bm→ basal membrane; ds→ desmosomes; gj→ gap junctions; mf→ microfilaments; m→ mitochondria; n→ nucleus; nu→ nucleolus; rer→ endoplasmic reticle; sj→ septata junction; v→ vesicles. (Bar = 1 μm)

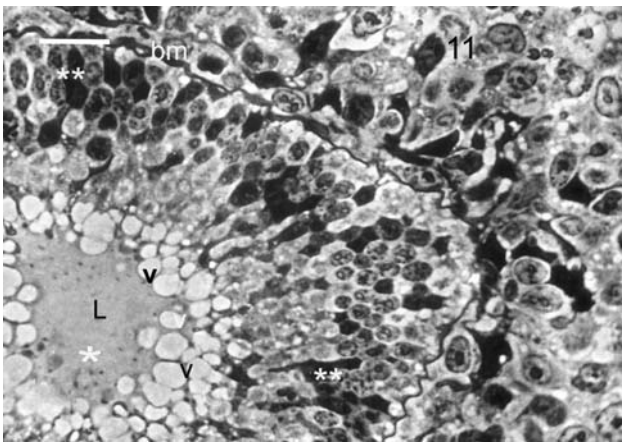


Fig. 11. Fifth instar nymph around the half of the instar period. Pedicel section at the apical zone. The secreted vesicles→ v are present at the periphery of the duct. Liberated product (*) In the lumen→ L. At the epithelium a large number of dense dark cells are present (**). Basal membrane→ bm. (Bar = 10 μm)

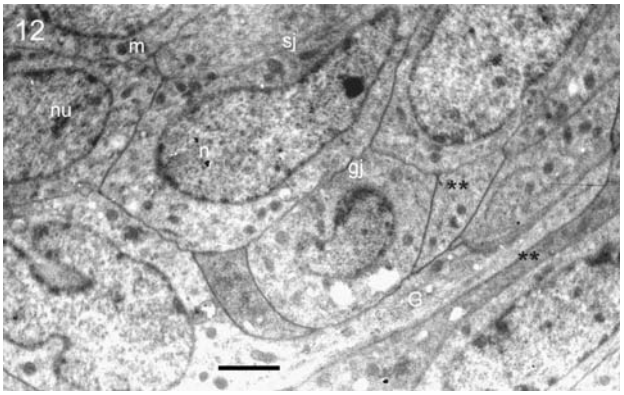


Fig. 12. TEM thin section micrograph of the pedicel epithelium from a just molted fifth instar nymph. Among the cells (gloves like) digitate processes, as well as cytoplasmic bridges (**) are easily seen. gj→ gap junctions; n→ nucleus; nu→ nucleolus; m→ mitochondria; G→ Golgi; sj→ septata junctions. (Bar = 0.5 μm)

Fifth instar nymphs at an advanced time after molting (40 to 50 days) present the before mentioned characteristics in a better designed mode (Fig. 16a - 16b - 16c). Taking in account the pedicel secretor activity, an important increase is found at the start of the period, between twentieth and thirtieth days, the secreted vesicles fill up the pedicel lumen proximal zone. Beyond that time their number diminished, and the volume that was occupied by them are now replaced by a fibrillar product. (Fig. 11 – 13 – 14b - 15).

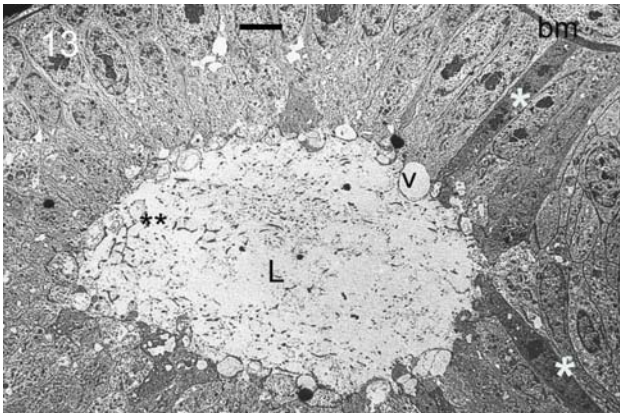


Fig. 13. Low magnifications TEM micrograph from the ovariole pedicel of a fifth instar nymph near to molt toward adult. The epithelium, lumen→ L filled with product (**) are shown. At the epithelium dense cells (*) are conspicuous at the zone adjacent to ovariole body. bm→ basal membrane (Bar = 1.5 μm)

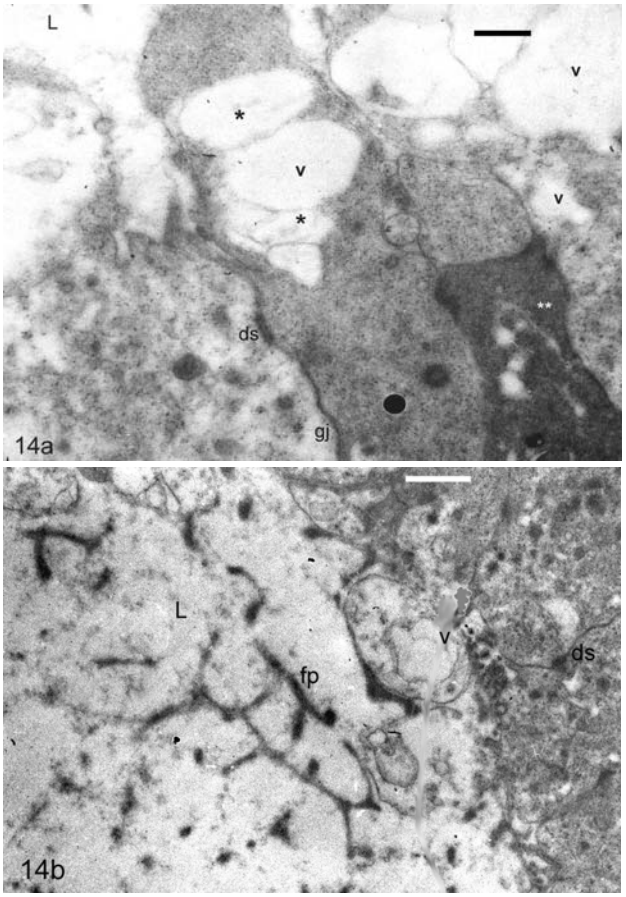


Fig 14*a*. Epithelium region where exocytosis can be observed with fibrils like elements (*) within the vesicles. ds→ desmosomes, gj→ gap junctions; v→ vesicles; (**)→ dense cell. (Bar = 0.25 μm)

14*b*. Detail of the fibril product→ fp that is liberated in the duct lumen→ L, by the epithelium cells. v→ vesicles; ds→ desmosomes. (Bar = 0.5 μm)

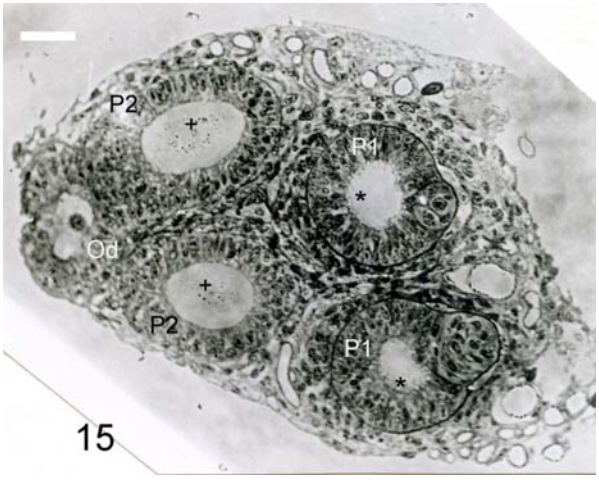


Fig. 15. Fifth instar nymph near to the molting period from a consecutive serial sections slides where nearly all the pedicel length has been trans-sectioned. Four ovarioles sections are present in this section; two of them at the cephalic or proximal level→ P_1, vesicle→ v, at lumen periphery (*), two at the medium-caudal zone→ P_2 with product at the lumen (+), the lateral oviduct→ Od, also present in the micrograph. (Bar = 25 μm)

The distal zone shows a lumen where vesicles are scarce, instead a dense filamentous product come into view, as a result the lumen center is filled by a bundle like microfilament structure (Fig. 14b).

The microfilaments which length is difficult to measure show when they are trans-sectioned a regular dense structure.

Comparing the secretion of each instars with specimens within 24 hours after molting to adult, the product found at the proximal pedicel zone is now composed by a homogeneous tubular product (Fig. 17). It is so regularly ordered that it seems to have crystal like features.

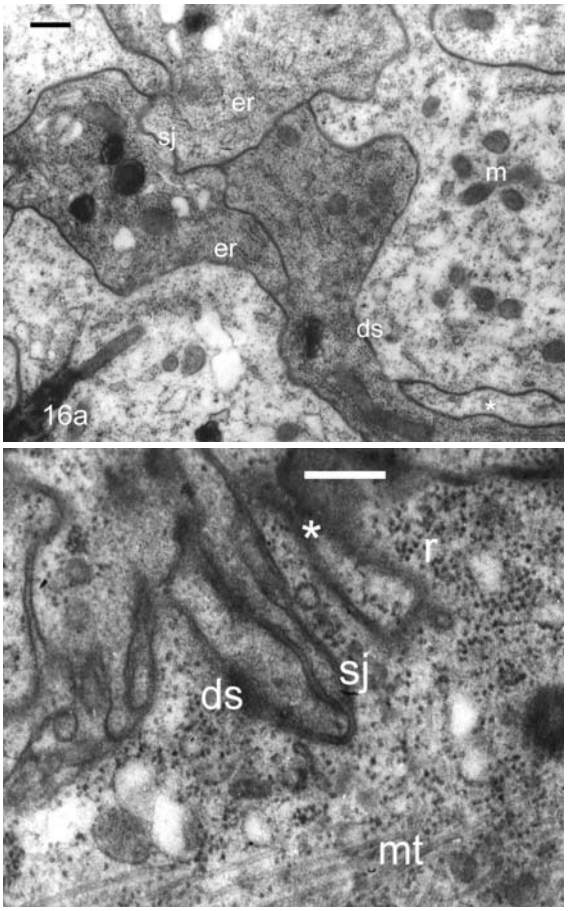
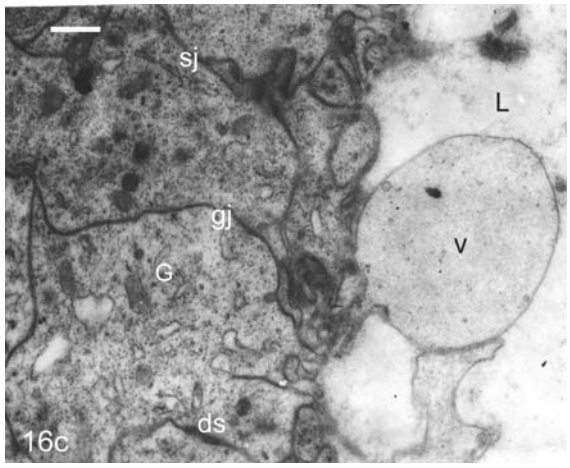


Fig 16. TEM micrograph of a fifth instar nymph near to the end of the period. Details of epithelium cell cytoplasm are shown.

16a) Interdigitation (*) [at botton wright]. Cell zone with profuse endoplasmic reticule→ er, m→ mitochondria, sj→ septata junction; ds→ desmosomes.

16b) The infolding of the plasma membrane (*); sj→ septata cell junctions; ds→ desmosomes; mt→ microtubules parallel oriented to each other, r→ ribosomes and polyribosomes are evident.

(Bar = 0.25 μm)



16c) On the surface of the pedicel lumen→ L an exocytic process is present, a large vesicle→ v with fibril-granular content is seen. Between cells septata junctions→ sj, as well as desmosomes→ ds with gap junctions→ gj can be appreciated; G→ Golgi. (Bar = 0.25 μm)

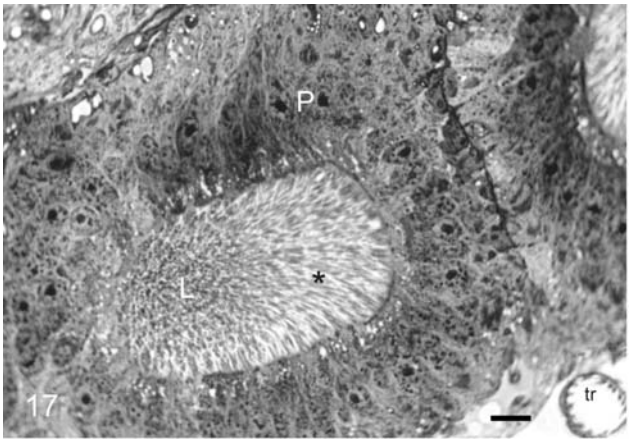


Fig. 17. Semi-thin section of a female adult just one day after molted. The section covers the apical P→ pedicel zone. The lumen→ L is filled with a reach content of profuse filament secretion (*); tr→ tracheole.

(Bar = 50 μm)

Together with the cytological analysis a morphometric study was performed taking the whole pedicel length as measure from the first instar to the fifth. The results of this measurement are presented at figure 4, shown previously. A morphometrical analysis has been performed in order to present a quantitave reference to the secretive activity. The light of trans-sections shows to be filled by vesicles, as well as by a variable number of other secretion “bits”.

The sections of the pedicel of nymph instars III, IV and V were analyzed, since in such nymph instars the filling process of proximal pedicel lumen surface by vesicles occurs. The results of these measurements are presented in fig 18 and 19. The data shows the proximal pedicel lumen and the total lumen surfaces respectively.

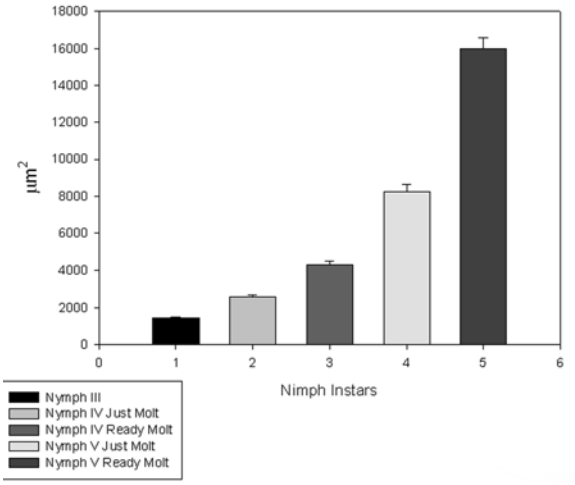


Fig. 18. Pedicel total luminal surface measured for nymph instars: third, just molted fourth, fourth ready to molt to fifth, just molted fifth, fifth ready to metamorphosis. Data are expressed as Mean ± s. e. m. in μm^2 x 10

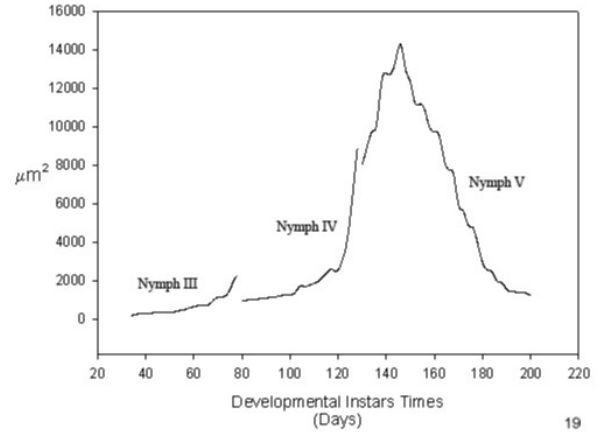


Fig 19. Total pedicel luminal surface occupied by discharged vesicles measured for nymph molted fifth, fifth ready to metamorphosis. Data are expressed as Mean ± s. e. m. in μm^2 x 10

DISCUSSION

After our study it should be mentioned that the ovariole stem of pedicel, and ovariole structures not much described for hemiptera insects' feminine gonads, at post embryo nymph instars reach a high degree of differentiation as well as cell activity. The third instar is the turning point when a continuous growth and differentiation occurs, is established up to the adult stage. When just molted samples of the third instar are analyzed a length increase is shown together with a secretor activity that in the proximal third of the pedicel is sustained through the instars up to metamorphosis. Such clear cut characteristics show a neat difference with the observed and described for other species where the pedicel cell differentiation seems to take place later, at the end of post embryo development when a high degree of cell differentiation in somatic cells also occurs [12–15].

Referring to genus *Triatoma* we revealed that at the forth instar ovariole pedicel length as well as cell activity increase conspicuously. About the length increase is maintained steadily during the forth instar and all over the fifth.

When the ultrastructural and functional stages of ovarioles cells is referred in the literature, they mention mainly the vitelarium and tropharium at the adult female only for any of the species study, [3,5,16–19]. The submicroscopic study was a careful comparison of each subsequent nymph instars. The variation in the mitochondria distribution as well as in the position and stages of nuclei and nucleoli, the endoplasmic reticule and the Golgi apparatus all them manifest cell synthesis activity. Cellular junction and bridges are mentioned in works for gametogenesis at their female adult stages [18–22], our studies found that in the first and second instars only septata type cell junctions are present; from the third instar some gap junction are observed; their frequency increases at forth and fifth instars, facts that can be taken as the occurrence of intracellular diffusion processes [23,24].

The pedicel shows in its structure the presence of cytoplasmic bridges, such structure should be underlined, since the literature refers as basic structures of germanium and vitelarium particularly related with germinal cells [25,26].

Morphological evidence that should be pointed is the secretory cell activity –clearly view even whit optical microscopy- that is found in the pedicel at the nymph instars. Such secretory product shows a constant and

steady increase from the third instar on, reaching a high accumulation of vesicles at the proximal pedicel third, feeling its tube light when samples of just molted fifth instar nymph are analyzed. Such vesicles secretion diminishes since that time on and particularly is very scarce –if still present– at the end of this last nymph instar, as is shown by the morphometrical study.

Our studies show that at the distal end of the pedicel duct, an accumulation of fibrillar product perhaps a metabolic transform of the vesicle contents, is particularly found when ready to molt fifth instar nymph samples are considered.

Also it is important to point that when the metamorphosis toward adult stage takes place, a new product of little tubes structure –that can be observed under a high magnification inmersion objective– is secreted into the pedicel proximal lumen. Pedicel shows different secretion types are found; we are able to think that different secretions might be the expression of a variety of functions, perhaps the differentiation of other regions, the oviduct as an example.

We consider the accepted concept that in incomplete metamorphosis insect the major growth, differentiation and secretory activity take place when the last instar nymph change to adult are due to the presence of circulating hormones [27–31] and with the experimental Lutz's works –1979, who affirm that ecdysone is the key factor in ovary differentiation. The observed process has a gradual pace starting at the third instar nymph reaching its maximum expression at the fifth instar nymph; might be coincident with certain level of hormones present, or with the reactive stage of them. Certainly it might be hypothesized too that the receptor competence for already present hormones change in the cells of pedicel epithelium, as observed by the different cell types.

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