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Comparative study of enzymes in testes and ovaries from adult *Dipetalogaster maximus* (Uhler) and *Triatoma infestans* (Klug) (Hemiptera: Reduviidae). Correlation with fine structural organization

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SUMMARY

Activities of hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PDH), fructose-6-phosphate kinase (F6PK), glutamate dehydrogenase (GlutDH), aspartate aminotransferase (AAT), malate dehydrogenase (MDH) and glycerol-3-phosphate dehydrogenase (GPDH) were determined in tissue extracts of testes and ovaries of adult *Dipetalogaster maximus* (Uhler) and *Triatoma infestans* (Klug) (Hemiptera: Reduviidae), insect vectors of Chagas disease. The fine structure organization of the same organs were studied by electron microscopy. Results allow the following inferences: in testes from both species, most of the glucose would be utilized through the glycolytic pathway. Amino acid catabolism for energy purposes appears to be unimportant. The number of mitochondria and the development of the rough endoplasmic reticulum in cells of the spermatogenic line indicate the occurrence of active oxidative metabolism and protein synthesis; in ovaries, levels of G6PDH indicate the existence of an active pentose pathway which would supply the NADPH required for fat and ecdysteroid synthesis. Amino acid catabolism appears to be relatively more important in ovary than in testis. Fat and glycogen are stored in follicular cells of *D. maximus*; oocytes of both species contain numerous fat droplets. Abundant mitocondria are present in follicular cells and oocytes. A well developed rough endoplasmic reticulum and free ribosomes are also conspicuous in these cells. The malate/aspartate H-transfer system seemed to be relatively more important than the glycerophosphate shuttle in ovaries as well in testes.

INTRODUCTION

Many members of the subfamily Triatominae are vectors of *Trypanosoma cruzi*, the etiological agent of American trypanosomiasis. This pathology, also known as Chagas' disease, is one of the most serious infectious disease in South America, with 17 million persons infected (Moncayo, 1993). Vectors of this disease are distributed throughout the American continent and some Caribbean islands, between latitudes 40º N and 56º S (Schofield, 1985).

Dipetalogaster maximus (Uhler), a sylvatic triatomine species which occupies mainly rocky habitats (Ryckman and Ryckman, 1967) is found in Mexico (Southernmost Baja California). *Triatoma infestans* (Klug) is the main vector of Cha-

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gas' disease throughout the southern half of South America. It is well known for its adaptation to domestic habitats (Lent and Wygodzinsky, 1979). Neither a reliable treatment for chronic chagasic patients nor a vaccine are available yet. So far, vector control is the most effective method to reduce the rate of infestation. The implementation of effective control methods requires a deep knowledge of the vector biology, biochemistry and physiology. We have recently studied enzymes involved in energy producing pathways of thoracic muscles of *D. maximus* and *T. infestans* in the latter stages of metamorphosis (Scaraffia *et al.*, 1997).

Knowledge of functional aspects of the organs involved in reproduction of these species may be useful for the development of control procedures. We have determined the activity of several enzymes related to carbohydrate and amino acid metabolism and correlated them with ultrastructure of adult testes and ovaries. Results are reported in this paper.

MATERIALS AND METHODS

Insects

Adult males and females of *D. maximus* and *T. infestans* were obtained from the insectary of the Centro de Referencia de Vectores, Servicio Nacional de Chagas, Córdoba, Argentina. Colonies were maintained at 28ºC ± 1ºC, under a relative humidity of 60 to 70% and a photoperiod of 6 hs light: 18 hs dark. Insects were fed on live chickens once every 2 weeks after the imaginal ecdysis. Four days after the second feeding, testes and ovaries were dissected out. Each sample weighing about 300 mg (wet weight) represented a pool of testes or ovaries. Tissue samples were homogenized in distilled water (1:5, W:V) using an Ultra-Turrax T 25 tissue disruptor. The tube with the preparation was placed in an ice bath during homogenization, which was performed in three 1-min pulses at 15-sec intervals. The suspensions were centrifuged at 20,000 g for 40 min (4ºC) and the supernatants used for enzyme and protein determinations.

Enzyme assays

Hexokinase (HK, EC 2.7.1.1). We followed the method of Bergmeyer *et al.*, (1974 a). The assay medium contained 45 mM Tris-HCl buffer pH 7.6, 185 mM D-glucose, 7 mM $MgCl_2$, 0.85 mM $NADP^+$ (monosodium salt), 0.60 mM ATP (disodium salt) and 0.65 U glucose-6-phosphate dehydrogenase.

Fructose-6-phosphate kinase (F6PK, EC 2.7.1.11). An adaptation of the method of Bergmeyer *et al.*, (1974 b) was utilized. The assay medium was: 70 mM Tris-HCl buffer pH 8.5, 1.40 mM $MgSO_4$, 4.30 mM KCl, 1.60 mM phosphoenol pyruvate (monosodium salt), 2.07 mM fructose-6-P (disodium salt), 1.08 mM ATP (disodium salt), 0.44 mM NADH (disodium salt), 30 U pyruvate kinase and 9.6 U lactate dehydrogenase.

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49). The technique described by Löhr and Waller (1974) was used. The assay medium was composed of 40 mM Tris-HCl buffer pH 7.6, 7 mM $MgCl_2$, 1.10 mM D-glucose-6-phosphate (disodium salt) and 0.82 mM NADP (monosodium salt).

Glutamate dehydrogenase (GlDH, EC 1.4.1.4). The method used by Cazzulo *et al.*, (1977) was followed. The assay medium contained 40 mM Tris-HCl buffer pH 7.6, 100 mM NH_4Cl, 0.90 mM EDTA, 0.17 mM NADH (disodium salt), 3 mM α-ketoglutaric acid (monosodium salt) and 1.40 mM ADP (potassium salt) .

Aspartate aminotransferase (AAT, EC 2.6.1.1). An adaptation of Bergmeyer and Bernt's method (1974) was utilized. The assay medium was 75 mM phosphate buffer pH 7.4, 12 mM α-ketoglutaric acid (monosodium salt), 50 mM L-aspartic acid (monopotassium salt), 0.17 mM NADH (disodium salt) and 5 U malate dehydrogenase.

Malate dehydrogenase (MDH, EC 1.1.1.37). The method of Yoshida (1969) was used. The assay mixture contained 100 mM Tris-HCl buffer pH 8.8, 1 mM oxalacetic acid and 0.17 mM NADH (disodium salt).

Glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8). The technique described by Fink and Brosemer (1975) was followed. The assay medium was composed of 50 mM MOPS buffer pH 6.6, 0.17 mM NADH (disodium salt) and 0.20 mM dihydroxyacetone phosphate (lithium salt).

The final assay volume was 3 ml for all enzymes, except for HK, GlutDH and GPDH, in which the final volume was 1 ml. Enzyme activities were determined in the supernatant by following the changes in absorbance at 340 nm in a Beckman DU 70 spectrophotometer with the cuvette compartment thermostabilized at 30ºC. In all the assays the reaction was started by addition of the enzyme

preparation, diluted to give an absorbance change between 0.050 and 0.250 per min and absorbance was read every 10 sec during 2 min. One unit of enzyme is the amount that utilizes 1 µmol of substrate per min in the assay conditions. The molar extinction coefficient of 6.22 cm^2/µmol was used to calculate NADH oxidation or NAD^+ and $NADP^+$ reduction. Activity is expressed as specific activity (U per mg protein) and U per g of wet tissue. Controls were performed in all determinations by omitting the substrate in the assay mixture. Values reported represent the mean ± standard error of 10 determinations on different samples.

Total Proteins

The protein contents were determined following the procedure of Smith *et al.* (1985).

Statistical analysis of data

Data are presented as means ± standard error of the mean. Significant differences between means were determined by the Student´s t-test. Excel program was used to perform one way analysis of variance. Duncan's test was applied for multiple comparisons of the means.

Electron microscopy

Adult testes and ovaries were fixed for 24 h at 4ºC by inmersion in a mixture of 4% glutaraldehyde, 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2 (Karnovsky, 1965). After fixation, the tissues were dissected, washed briefly in distilled water and treated with 1% osmium tetroxide for 1 h at room temperature. After dehydration in a series of cold acetone solutions, the tissues were embedded in araldite and sliced with a Jeol Jum-7 ultramicrotome. Thin sections were mounted on naked 200 mesh copper grids, contrasted with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 101 electron microscope.

RESULTS

In testes and ovaries from *D. maximus* and *T. infestans* the enzymes investigated showed no significant differences in activity between species, except for hexokinase (HK), which was lower in *D. maximus* (Tables I and II).

Testes

Activities of fructose-6-phosphate kinase (F6PK) in *D. maximus* and *T. infestans*, either expressed in U/mg protein or U/g wet tissue, were higher than that of glucose-6-phosphate dehydrogenase (G6PDH).

HK activity was lower than F6PK activity in *D. maximus*; in contrast, HK was higher than F6PK in *T. infestans*.

Table I
Enzyme activities of testes from *Dipetalogaster maximus* and *Triatoma infestans*

Enzyme	*Dipetalogaster maximus*		*Triatoma infestans*	
	U/g Tissue	U/mg Protein	U/g Tissue	U/mg Protein
HK	0.65 ± 0.06 *	0.02 ± 0.001 *	3.66 ± 0.42	0.07 ± 0.008
F6PK	1.45 ± 0.16 ns	0.03 ± 0.004 ns	1.69 ± 0.26	0.03 ± 0.005
G6PDH	0.37 ± 0.05 ns	0.01 ± 0.001 ns	0.46 ± 0.17	0.01 ± 0.003
GlutDH	0.70 ± 0.04 ns	0.02 ± 0.001 ns	0.75 ± 0.05	0.02 ± 0.001
AAT	3.14 ± 0.18 ns	0.07 ± 0.004 ns	3.39 ± 0.49	0.07 ± 0.010
MDH	39.52 ± 2.82 ns	0.92 ± 0.067 ns	37.56 ± 0.55	0.75 ± 0.011
GPDH	1.05 ± 0.05 ns	0.02 ± 0.001 ns	0.90 ± 0.05	0.02 ± 0.001

Values represent means ± S.E. of determinations on 10 different samples. Amounts of total soluble protein were: 42.73 ± 1.11 mg/g of wet tissue in *D. maximus* and 50.37 ± 1.73 mg/g of wet tissue in *T. infestans.* *Indicates significant difference with *T. infestans;* $p<0.001$; ns: not significant with *T. infestans* (Student's test). In *D. maximus* F6PK activity is significantly different from HK, G6PDH and GlutDH ($p< 0.01$). In *T. infestans* F6PK activity is lower than HK activity but F6PK activity is higher than G6PDH and GlutDH activities ($p< 0.01$). In each species GPDH activity is lower than AAT and MDH activities ($p<0.01$). ANOVA and Duncan's test.

Table II

Enzyme activities of ovaries from *Dipetalogaster maximus* and *Triatoma infestans*

Enzyme	*Dipetalogaster maximus*		*Triatoma infestans*	
	U/g Tissue	U/mg Protein	U/g Tissue	U/mg Protein
HK	0.95 ± 0.08 *	0.01 ± 0.001 *	1.95 ± 0.06	0.03 ± 0.001
F6PK	1.69 ± 0.31 ns	0.02 ± 0.003 ns	1.45 ± 0.17	0.02 ± 0.003
G6PDH	1.91 ± 0.12 ns	0.02 ± 0.002 ns	1.37 ± 0.16	0.02 ± 0.002
GlutDH	2.40 ± 0.05 ns	0.03 ± 0.001 ns	2.02 ± 0.05	0.03 ± 0.001
AAT	3.86 ± 0.42 ns	0.05 ± 0.005 ns	4.15 ± 0.26	0.06 ± 0.004
MDH	45.99 ± 3.21 ns	0.58 ± 0.041 ns	30.86 ± 1.86	0.48 ± 0.029
GPDH	2.76 ± 0.30 ns	0.04 ± 0.004 ns	2.40 ± 0.08	0.04 ± 0.001

Values represent means ± S.E. of determinations on 10 different samples. Amounts of total soluble protein were: 78.97 ± 2.10 mg/g of wet tissue in *D. maximus* and 65.21 ± 1.84 mg/g of wet tissue in *T. infestans*.*Indicates significant difference with *T. infestans*; $p < 0.001$; ns: not significant difference with *T. infestans* (Student's test). In *D. maximus* HK activity is significantly lower than F6PK, G6PDH and GlutDH activities ($p< 0.05$). There are no significant differences between F6PK and G6PDH activities. In *T. infestans* F6PK activity is lower than HK and GlutDH activities ($p< 0.05$). F6PK and G6PDH activities are not significantly different. In each species GPDH activity is lower than AAT and MDH activities ($p< 0.01$). ANOVA and Duncan's test.

The level of F6PK was twice as high as glutamate dehydrogenase (GlutDH) in both species.

Enzymes involved in the malate-aspartate system for hydrogen transfer to mitochondia (aspartate aminotransferase, AAT, and malate dehydrogenase, MDH) showed higher activity than glycerophosphate dehydrogenase (GPDH), which participates in the glycerophosphate shuttle (Table I).

Ovaries

Fructose-6-phosphate kinase showed similar activity to G6PDH. At variance with *T. infestans*, HK activity in *D. maximus* was lower than that for F6PK.

The level of GlutDH was higher than that for F6PK in both species. AAT and MDH activities were greater than that of GPDH (Table II).

Protein

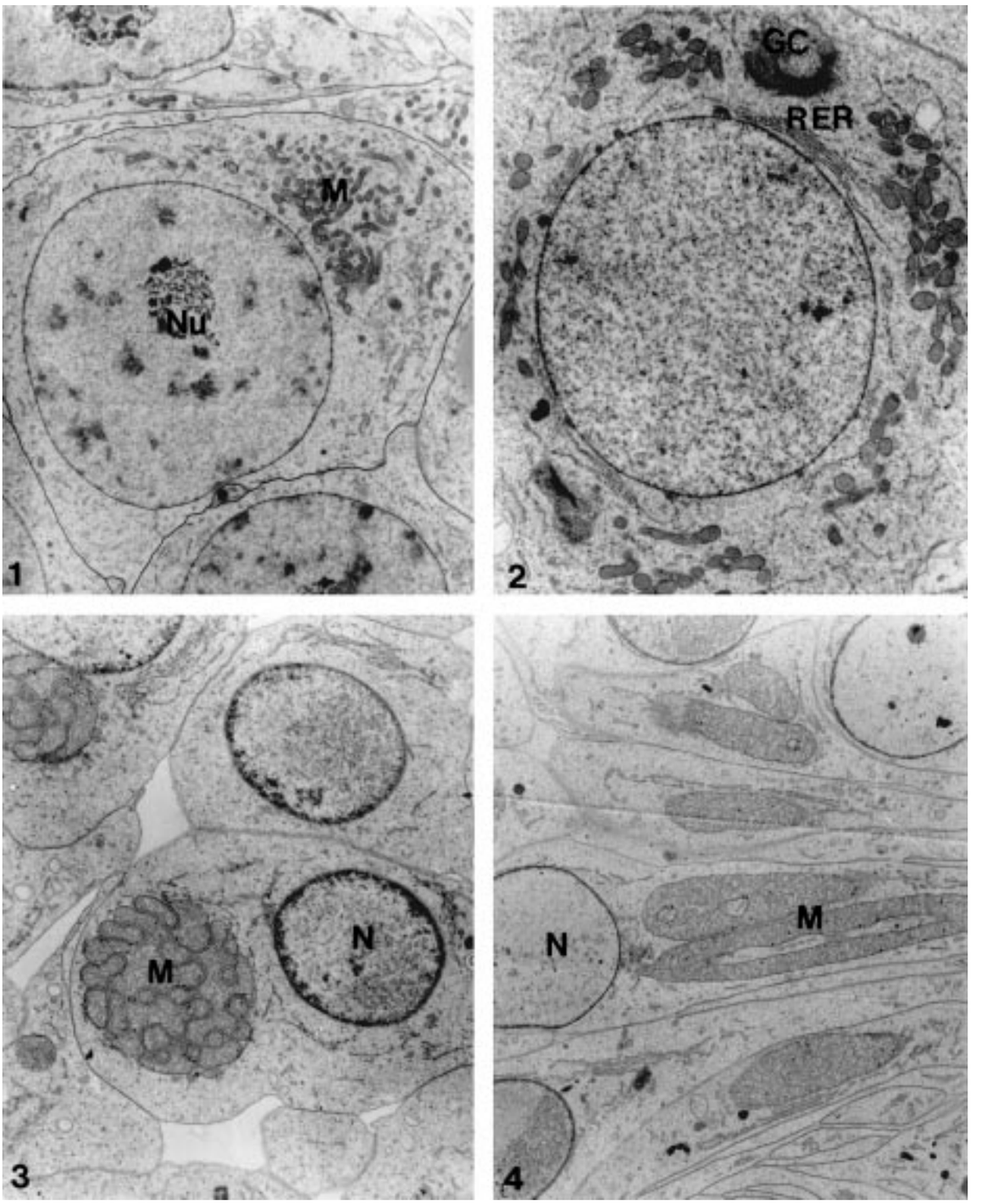
Total soluble protein values, expressed in mg per g of wet tissue, were 42.73 ± 1.11 and 50.37 ± 1.73 for testes from *D. maximus* and *T. infestans* respectively (Table I). In ovaries the values were 78.97 ± 2.10 in and 65.21 ± 1.84 for ovaries from *D. maximus* and *T. infestans* respectively (Table II).

Electron microscopy

Testes. In testes of *D. maximus* and *T. infestans*, the shape, size and distribution of mitochondria changed strikingly along the spermatogenic cell line (Figs. 1-4). In early stages of spermatocytes, mitochondria are small, oval or round (Fig. 1). In later stages, spermatocytes show increasing numbers of larger mitochondria which spread throughout the cytoplasm (Fig. 2). Besides, the spermatocytes exhibit abundant rough endoplasmic reticulum (RER) cisternae and typical Golgi complexes.

Spermatids present characteristic mitochondria clustered into a single large mitochondrion, or mitochondrial derivative (nebenkern) (Fig. 3). In this giant structure, the fusion of the mitochondrial subunits continues until two large interlocking

Figs. 1/4 - (1) Spermatocyte from *D. maximus* testes. Small mitochondria (M) are clustered in a perinuclear area. Nuclei contain big nucleolus (Nu). X 10, 500. **(2)**Spermatocyte from *T. infestans*. Numerous predominantly oval mitochondria appear intermingled with RER cisternae and free ribosomes. GC: Golgi complex. X 10, 500. **(3)**Spermatid of *T. infestans.* A large mitochondrial derivate (M) occurs in a paranuclear position. In the nucleus (N), heterochromatin form a ring against the nuclear membrane. X 10, 500. **(4)**Late spermatid of *D. maximus*. Two separated elongated mitochondria (M) show a rich cristae development. X 10, 500.



mitochondrial masses are formed (Fig. 4). In the next stage, the two interwoven nebenkern mitochondria start to elongate and stretch along the base of the future flagellum.

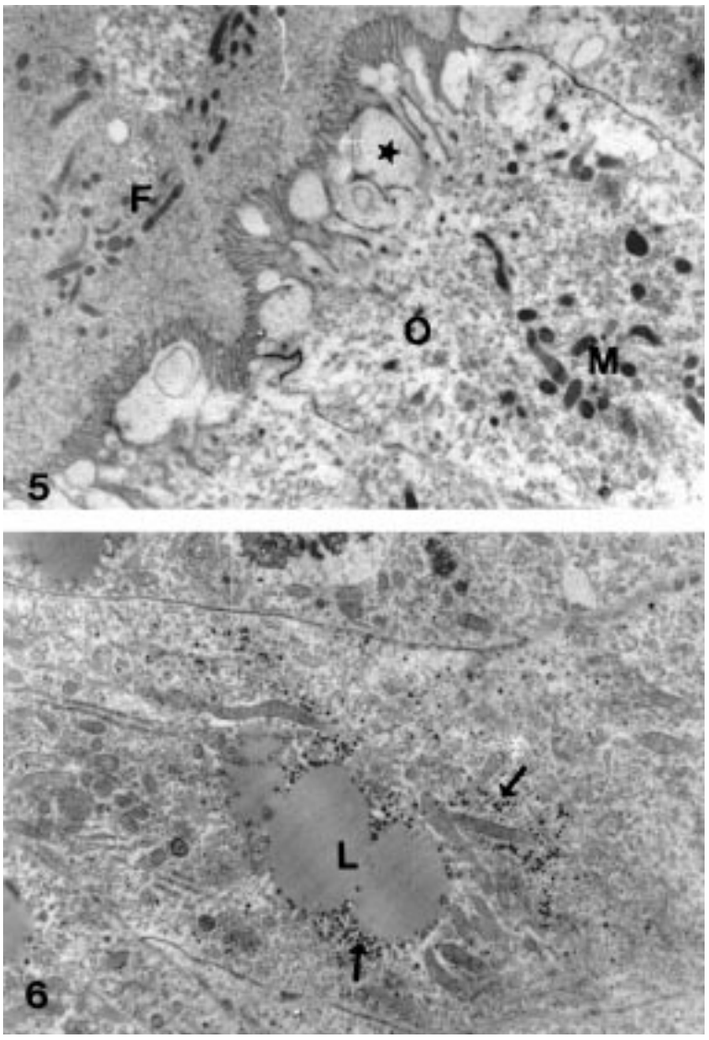
Ovaries. Follicular cells exhibit elongated mitochondria intermingled with abundant membranes of RER and free ribosomes (Fig. 5). Occasionally they may contain lipid droplets with associated glycogen particles (Fig. 6)

In oocytes from both species, mitochondria are long and slender and frequently associated with abundant lipid droplets (Fig. 7). Lipid content varies according to the oocyte stage. Abundant free ribosomes fill up the rest of the oocyte cytoplasm.

DISCUSSION

Observations at the ultrastructural level and determinations of enzyme activities of gonads from adult *Dipetalogaster maximus* and *Triatoma infestans* allow some inferences on the relative importance of different metabolic pathways operating in those organs.

In testes, ratios of activities of fructose-6-phosphate kinase over glucose-6-phosphate dehydrogenase (F6PK/G6PDH) would suggest that, in both species, a greater proportion of the glucose metabolized follows the glycolytic instead of the pentose pathway. The high level of hexokinase,



Figs. 5/6 - (5) Follicular cell (F) and oocytes (O) of *T. infestans* separated by interdigitation area (*). Oocyte mitochondria (M) are small and appear frequently in the periphery. X 10,500. **(6)**Lipid droplets (L) associated with glycogen particles (arrowheads) are present in some follicular cells from *D. maximus.* X 10, 500.

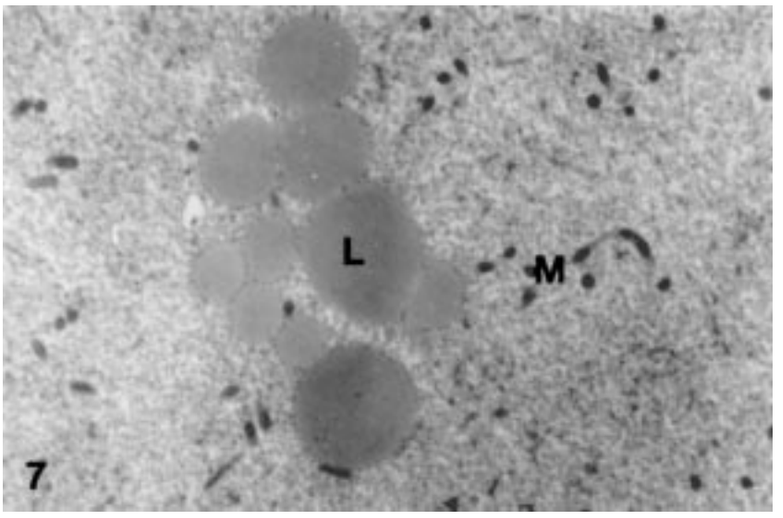


Fig. 7 - Oocyte of *D. maximus* ovaries containing abundant lipid droplets (L) in contact with thin and elongated mitochondria (M). The remaining cytoplasm contains numerous ribosomes. X 10, 500.

specially in *T. infestans*, indicates that glucose supplied by the hemolymph would be a major fuel for testis rather than that produced by endogenous glycogenolysis.

In both species, values of glutamate dehydrogenase (GlutDH) would indicate that amino acid catabolism for energetic purposes appears to be unimportant. In contrast, flight muscles from the same insects show a much higher GlutDH activity: 1.61 U/mg for *D. maximus* and 0.87 for *T. infestans* (Scaraffia *et al.*, 1997) suggesting a larger contribution of amino acid catabolism to the production of energy in muscles than in testes.

The abundance of mitochondria in cells of the spermatogenic line would reflect an intense respiratory activity. Changes in shape, size and distribution of these organelles are interesting. They are similar to those described by Phillips (1970) for other species. Particulary abundant are the mitochondria in spermatocytes, which are organized in large clusters. Up to now we have no clues on the functional significance of these mitochondrial rearrangements. Striking changes also occur in mitochondria of higher animals during sperm differentiation from spermatocytes up to the formation of the mitochondrial sheath of the mid-piece of spermatozoa (André, 1962). However, the highly structured mitochondrial clusters shown here are not observed in higher species.

Aspartate aminotransferase (AAT) and malate dehydrogenase (MDH) activities compared to that of glycerophosphate dehydrogenase (GPDH) would indicate that the malate/aspartate shuttle would be more active than the glycerophosphate system for the transfer of reducing equivalents into the mitochondria.

Spermatocytes and spermatids of both species present a well developed rough endoplasmic reticulum and abundant free ribosomes, reflecting an important protein synthetic activity.

In ovaries, follicular cells of *D. maximus* exhibited prominent fat droplets surrounded by glycogen. Similar observations were reported by Lutz and Huebner (1982) in prefollicular cells of *Rhodnius prolixus.* It appears then, at variance with testis, that glycogen and fats represent significant fuel stores in the ovary. In *D. maximus*, the higher activity of F6PK compared with HK probably indicates a relatively larger use of glucose from glycogen degradation.

Activities of HK in ovary, as well as in testes of *T. infestans* are much higher than those for *D. maximus*. This difference cannot be explained with the available data. It would appear that the capacity to utilize free glucose is higher in the cells of *T. infestans* ovaries and testes than of *D. maximus*.

In both species, the ratios of activities F6PK/G6-PDH for ovaries are much lower than those of testes, suggesting that an important proportion of the glucose utilized may be diverted to the pentose pathway, thereby providing the required NAPDH for the synthesis of fatty acids and ecdysteroids.

The production of ecdysteroids in ovary has been reported for many species (Grieneisen, 1994). The site of ecdysteroid synthesis has been localized in follicular cells of telotrophic ovaries from *Tenebrio molitor* (Laverdure *et al.*, 1977) and *Rhodnius* (Ruegg *et al.*, 1981, 1982) and the follicular epithelium of *Locusta migratoria* y *Nauphoeta cinerea* (Goltzené *et al.*, 1978; Zhu and Lanzrein, 1984).

In ovaries, the activity of GlutDH is higher than that in testes, indicating that the ovary would utilize amino acids as energy source to a larger degree than the testis.

Mitochondria are abundant in follicular cells and oocytes. The relative activities of aspartate aminotransferase and malate dehydrogenase with respect to GPDH in ovaries follow the same trend as in testes. This suggests that the malate/aspartate shuttle in ovaries as well as in testes would be more active than that of glycerophosphate, at variance with our finding in muscles from the same insects (Scaraffia *et al.*, 1997).

It has been demonstrated that follicular cells play an important role in the synthesis of yolk components. Among these are glycoproteins, phospholipds and triacylglycerides (Wigglesworth, 1972). Applying cytochemical techniques, Verma *et al.* (1972) described the occurrence of fatty and compound yolk in oocytes of *Aspongopus obscurus*. They comprise unsaturated neutral fats synthesized in oocytes. The compound yolk (CY), constituted by carbohydrates, protein and RNA has a dual origen: CY1 is synthesized de novo in oocytes, while CY2 is produced by modifications of yolk precursor substances incorporated into the follicular epithelium by pinocytosis.

Our studies show that enzymes and structures involved in the synthesis of triacylglycerides, phospholipids and proteins are present in ovaries of *D. maximus* and *T. infestans*. The pentose pathway would be the NADPH provider; the GPDH producing glycerophosphate from phospho-dihydroxyacetone, would supply a precursor for phospholipids and triacylglycerides. The conspicuous rough endoplasmic reticulum and free ribosomes in follicular cells and oocytes reflect an active protein synthesis. There is a higher content of total soluble protein in ovaries than in testes.

Results reported here are the first study on the metabolic capabilities of reproductive organs of two vectors of Chagas disease. We hope that a better understanding of these insects' biochemistry will provide more rational bases for the design of control methods.

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