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RESEARCH

Histopathological Changes in Third-Instar and Adult *Anastrepha ludens* (Diptera: Tephritidae) After in vitro Heat Treatment

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ABSTRACT. The Mexican fruit fly, *Anastrepha ludens* Loew (Diptera: Tephritidae), is one of the most harmful pests of mango causing direct damage by oviposition on the fruit pulp. Mango for export is subjected to hydrothermal treatment as a quarantine method for the control of this pest, but exposure to heat for long periods of time reduces considerably the quality and shelf-life of treated fruit. The aim of this work was to study morphological changes of third-instar larvae and adults of *A. ludens* after in vitro exposure to high temperature at sublethal times. A heating block system was used to expose larvae at 46.1°C for 19.6 and 12.9 min, producing 94.6 and 70% mortality, respectively. Treated larvae were processed for optical microscopy. A fraction of surviving treated larvae was separated into containers with artificial diet to allow development into adults. Adult sexual organs were dissected and processed for transmission electron microscopy analysis. Results showed that 94.6% of the treated larvae died at 46.1°C for 19.6 min and none of the surviving larvae eclosed to adulthood, as they developed as malformed puparia. For the in vitro treatment at 46.1°C during 12.9 min, 70% of the treated larvae died and only 3.75% reached the adult stage, but ultrastructural damage in the male testes and in the female ovaries was observed. Additionally, 11.1% of the adult flies from the in vitro treatment also showed wing malformation and were incapable of flying. The analysis showed that surviving flies were unable to reproduce.

Key Words: *Anastrepha ludens*, heat treatment, ultrastructure, heating block system

Several species of fruit flies (Diptera: Tephritidae) have great economic importance and are considered as key pests that most adversely affect the production and marketing of fruits and vegetables worldwide. The most important genera in the Americas are *Ceratitis* MacLeay, *Rhagoletis* Loew, *Toxotrypana* Gerstaecker, and *Anastrepha* Schiner (Castaneda et al. 2010). *Anastrepha* is a genus of Tephritidae with the largest diversity of species in the Neotropical Region with more than 250 described species (Norrbom and Korytkowski 2011). *Anastrepha ludens* Loew (1873) (Diptera: Tephritidae) is considered the most serious insect pest of mango (*Mangifera indica* L., 1753) and several species of citrus fruit in regions of Mexico, Central America, and the Caribbean (Aluja et al. 1996, Quiroga et al. 1996, Thomas and Loera-Gallardo 1998, Castaneda et al. 2010, Norrbom and Korytkowski 2011). In Mexico, the mango-growing regions from Sinaloa to Chiapas States (including Nayarit, Jalisco, Colima, Michoacán, Guerrero, and Oaxaca) cover an area of 152,284 ha, with an average annual production of 9.86 ton/ha, and a total value of $323 million USD, respectively (Servicio de Información Agroalimentaria y Pesquera [SIAP] 2013).

There are several approved quarantine treatments for export of Mexican mango, such as hydrothermal treatments using heated air, hot water, or irradiation. Although mangoes grown in fruit-fly-free areas of northern Mexico do not need treatment, some of the mango varieties exported from Mexico to the United States, Japan, and some European countries are required to undergo hydrothermal treatment, heated air, or irradiation to prevent potential infestation with immature stages of *A. ludens* (United States Department of Agriculture/Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación [USDA/SAGARPA] 2010). Hydrothermal treatment, the most widely addopted procedure, requires fruit immersion in water at 46.1°C for 65–110 min depending on the fruit weight. The pulp temperature must be at least 45°C by the end of the hot-water immersion treatment in accordance with (United States Department of Agriculture/Animal and Plant Health Inspection Service/Plant Protection and Quarantine [USDA/APHIS/PPQ] 2013) guidelines to kill eggs and larvae of *A. ludens*, *Anastrepha obliqua,* and *Anastrepha serpentina* and in accordance with export protocols between Mexico and Japan (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación [SAGARPA] 2009), and Mexico and the United States (USDA/SAGARPA 2010, USDA/APHIS/PPQ 2013).

Mortality caused by heat has been determined in some insect species using the heating block system (HBS, Ikediala et al. 2000). This system has been widely applied to different biological models for establishing the parameters of the thermal death kinetics for larvae such as *Cydia pomonella, Amyelois transitella,* and *Plodia interpunctella*. A diversity of temperatures from 46.1 to 54°C and heating rates of 1, 10, and 18°C min^{-1} have been used (Wang et al. 2002, Johnson et al. 2004). The thermal death kinetic model for eggs and larvae of the red flour beetle *Tribolium castaneum* has been applied using temperatures ranging from 48 to 52°C, at a heating rate of 15°C min^{-1} (Johnson et al. 2004). Other studies have been conducted to determine the thermal death kinetic parameters for *Ceratitis capitata*, using temperatures ranging from 46 to 52°C at a heating rate of 15°C min^{-1} (Gazit et al. 2004); and for the third instars of *A. ludens* using temperatures from 44 to 50°C at a heating rate of 1.5°C min^{-1} (Hallman et al. 2005). However, there is little information about the cellular and molecular mechanisms that allow the survival of some larvae after the thermal stress. In particular, little is known about the ultrastructural changes of the reproductive organs in the surviving

insects. The objective of this work was to describe the histopathological changes of third-instar larvae and adult flies of *A. ludens* after in vitro thermal treatment at sublethal times using an HBS.

Materials and Methods

Larval Rearing. Larvae of the Mexican fruit fly developed from eggs from a mass-reared colony seeded in artificial diet according to methods described by Stevens (1991) and Dominguez et al. (2010) at the Moscafrut Facility at Metapa de Dominguez, Chiapas, Mexico. Larvae were deposited in plastic trays with diet and sealed with a mesh to allow air to pass through, sprayed every day with water and placed in a room at 26°C, 80% RH, and a photoperiod of 14:10 h (L: D).

Starting from first instars, larval development was monitored daily using a stereomicroscope (Motic, SMZ-140-N2GG, Hong Kong, China). Morphological and morphometrical characteristics, such as size, color, and mouth hook formation were monitored. The second instar was reached between the third and the sixth day of growth, when the mouth hook was half dark, half light. Besides, the gut was full because at this stage its main function is eating. The third instar was achieved between 6 and 9 d of growth, when the mouth hook was black. The larva and anterior spiracle were well developed, showed high mobility, and white color. Third-instar larvae were used for the in vitro thermal treatment.

Heating Block System. An HBS built as described by Ikediala et al. (2000) was used to heat the third-instar larvae. Two aluminum blocks (25 by 25 by 2 cm) and an intermediate thin aluminum slab (25 by 25 by 0.3 cm) with four square holes (5 by 5 cm) located symmetrically near to the center fit together thus forming four closed square chambers, where the larvae were placed. Heat was supplied by electric heating pads attached to the upper and lower surfaces of each plate, connected to a voltage regulator (ISB Sola Basic, microvolt Inet, Mexico) and controlled by a standard rheostat.

Larva Thermal Treatment. A total of 40 third-instar larvae along with 7 ml of rearing medium were placed in each of the four chambers of the HBS. The larvae from two of the chambers were used in the mortality trial and the remainder for the histological study. The in vitro experiment was performed at 46.1°C applying 120 V to the HBS and replicated three times. Before initiation of each trial, the HBS was set at 25°C; later, the upper plate was removed to place four T-type thermocouples (with an accuracy of ± 0.5°C), which were connected to a data acquisition module (Omega, OMB-DAQ-55, Stamford, CT) for temperature recording. The module was calibrated at the set points of 0 and 100°C.

Temperature was recorded every 3 s, and the treatment time started when the target temperature (46.1°C) was reached, which was held for the selected exposure times (19.6 and 12.9 min). Control larvae were treated the same way at room temperature but without heat. For each replicate, 40 out of 80 heated larvae were immediately fixed with formaldehyde (10%) in a phosphate buffer solution (PBS), pH 7.2. Half of the treated larvae were fixed in Bouin solution and the remainder 20 larvae with Carnoy fixer, and then processed for histopathology using an optical microscope. For survival analysis, 80 larvae were treated at 46.1°C for 19.6 and 12.9 min. Larvae were transferred to different plastic containers with pupariation medium (sterile vermiculite) to allow development to the adult stage. The sexual organs of the surviving adult flies were dissected. For this purpose, they were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, and analyzed by transmission electron microscopy.

Optical Microscopy. From each trial, 40 surviving third instars were taken and immediately fixed with 10% formaldehyde in PBS, pH 7.2, as well as with the Bouin and Carnoy solution for 2 h at room temperature. Complete specimens were placed in small filter paper envelopes and washed with water for 4 h. Next, the specimens were dehydrated using increasing concentrations of ethanol: 70, 80, 96, 100% and absolute xilol for 1 h. Finally, they were embedded in paraffin blocks (Fuentes et al. 2005). Histological cuts of 5 µm were performed with a microtome (RM2135, Leica, Germany) and dyed with hematoxylin–eosin, according to previously described protocols (Rodríguez-Santiago 2002). Slices were observed with an optical microscope Primo Star (Zeiss, Germany) with lens of 40× and 100× .

Transmission Electron Microscopy. After reaching sexual maturity, the sexual organs of flies from the in vitro thermal treatment were dissected and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature. Then, the specimens were postfixed with 1% osmium tetraoxide. Samples were dehydrated by immersion for 10 min using increasing concentrations of ethanol: 50, 70, 90, and 100%, and finally with propylene oxide for 20 min. Samples were embedded in epoxy resin (EMBed-812, EMS, USA) and polymerized at 60°C for 48 h in a stove (Gravity Convection Oven DX300, Yamato, Japan) (Valdez 2001, Brauer and Viettro 2003). The blocks were cut using an ultra microtome (Ultra Cut E, Richard-Joung, Austria), and ultrafine 70-nm-thick cuts were placed in 200 mesh copper grids. They were dyed with uranil acetate and lead citrate to be finally observed in the transmission electron microscope (TEM1400, Jeol, Japan).

Results

Histological Study of Third-Instar Larvae After *In Vitro* Thermal Treatment at 46.1°C. Three trials with 80 third-instar larvae each were performed and exposed at 46.1°C for 19.6 min. A 94.6% mortality was achieved, and from the 240 treated larvae, only 13 survived and pupated (Table 1). Nevertheless, these puparia did not hatch at day 17 as expected for untreated larvae, and therefore, they did not continue development to the adult stage, as they developed into malformed puparia.

The in vitro thermal treatment of larvae for 12.9 min generated 70% mortality. The histological study of the surviving larvae (30%) showed very noticeable changes in cellular morphology when compared with control larvae. Cytoplasm with abundant vacuolization and alterations of the nuclear membrane were observed. The nuclei of treated larvae showed diffuse granulate chromatin in all cells of several tissue regions, which was not observed in cells of untreated larvae used as control (Fig. 1C and D). In larvae with 94.6% mortality, changes in nuclei and cytoplasm were more evident (Fig. 1E and F). In this trial, only an average of 24 of 80 larvae survived, and all of them developed into the puparium stage after the expected 17 d. Only an average of three adult flies eclosed in each experiment (Table 1). A total of nine adult flies (eight males and one female) were observed. However, one adult male (11.11%) from the first trial resulted with external malformation, when compared with the adult control fly (Fig. 2).

Transmission Electron Microscopy. Ten days after eclosion, when flies reached their sexual maturity, the sexual organs of the three flies from the first trial (46.1°C for 12.9 min), including the malformed fly, and those of three control flies were dissected. The ultrastructural analysis of the sexual organs in the treated and untreated adult flies revealed very noticeable morphological differences in cells of primary follicles in the ovaries. A hypertrophy mainly in the external layer of the primary follicles, as well as in the basal region of the ovaries could be observed (Fig. 3C and D). A homogeneous unique long follicle was observed in the testes of untreated flies, which was surrounded by a peritoneal sheath formed by a layer of muscles, a basement membrane, a follicular epithelium, and a net of tracheoles. Normal cells with large nuclei were observed in the germarium zone of testes. However, follicular cell destruction was evident in the basement membrane and follicular epithelium of testes from treated male flies (Fig. 4C and D). Damage was

Table 1. Mortality of larvae treated at 46.1°C

Time and mortality	Death larvae	Puparied	Flies emerged
19.6 min and 94.6%	78 (97.5%)	2	0
	75 (93.75%)	5	0
	74 (92.5%)	6	0
12.9 min and 70%	57 (71.25%)	23	3
	56 (70%)	24	3
	55 (68.75%)	25	3

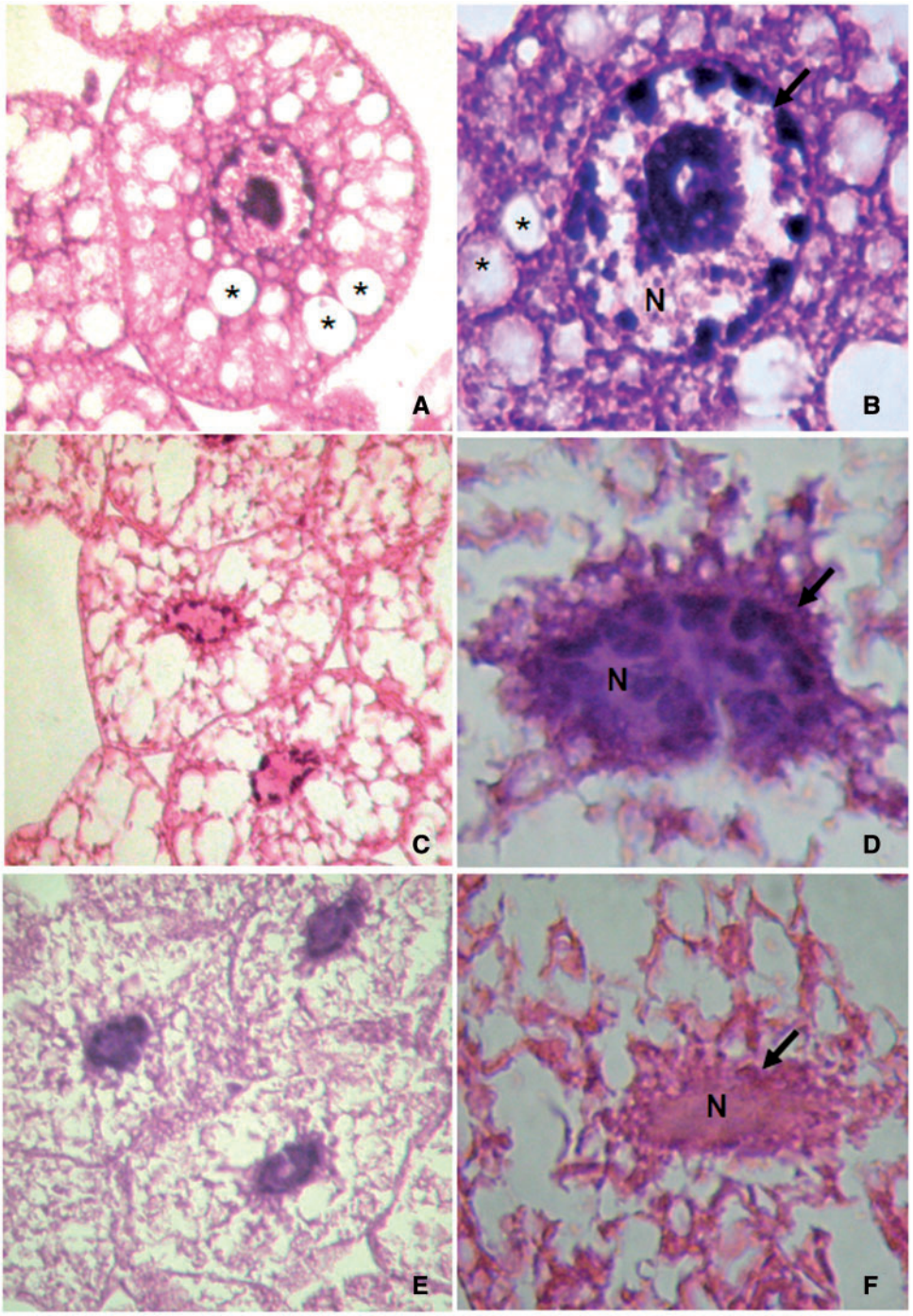

Fig. 1. Histological hematoxylin–eosin stains of untreated and thermal treated larvae of *A. ludens*. (A, B) Abundant liposomes (asterisks) and normal nucleus with uniform chromatin (arrow) in cells of the posterior part of untreated larvae used as a control. (C, D) Nuclear and cytoplasm destruction with abundant vacuolization in cells of the same region of larvae exposed at 46.1°C for 12.9 min that rendered 70% mortality. (E, F) Cells of treated larvae at same temperature during 19.6 min (94.6% mortality), where cellular changes as in nuclear membrane (arrows) as in cytoplasm is more evident. Amplifications: A, C, and E = ×40. B, D, and F = ×100. N, nucleus.

mainly observed in the germarium zone where important cellular atrophy was described.

Discussion

The conventional procedures of fixation with 3% and 5% formaldehyde and embedding in paraffin did not produce good histological preparations, perhaps due to the keratinized cover of the larvae. Modifications were made in the fixation process using concentrations of 2.5, 3, 5, and 10% formaldehyde in PBS, pH 7.2, as well as the Carnoy and Bouin fixers for 2 h at room temperature. The fixation for best conservation of cellular structures of the larvae was obtained using 10% formaldehyde, and no differences were observed between the Carnoy and Bouin fixers (Fuentes et al. 2005).

For many years, fresh commodities intended for human consumption have required safety regulation processes to ensure phytosanitary harmlessness. Fresh fruit has been subjected to physical treatments, such as heat exposure or ionizing radiations (Lopez-Muñoz 2007), as well as chemical treatments with agents like ethylene bromide.

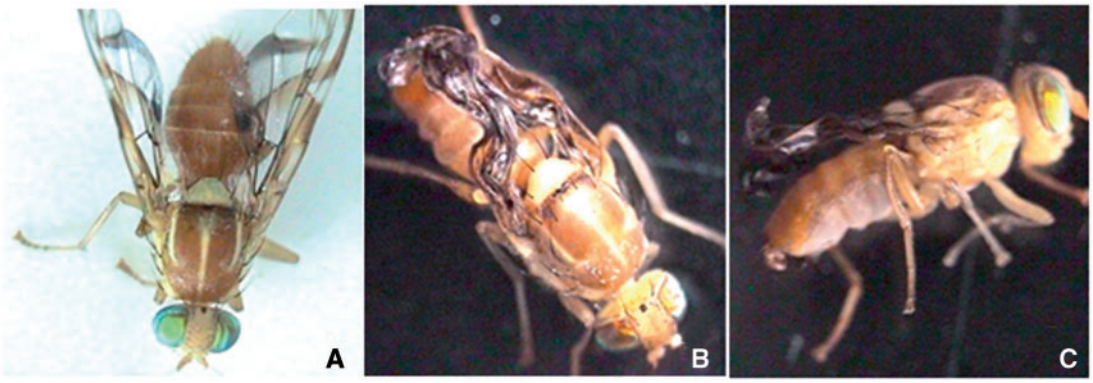

Fig. 2. Morphological alterations of the adult of *A. ludens* that survived to the 46.1°C for 12.9-min treatment that produced 70% mortality. (A) Typical morphology of a healthy fly without treatment. (B, C) Adult fly that survived to the in vitro treatment showing malformations in the wings. Amplifications: A–C = ×4.

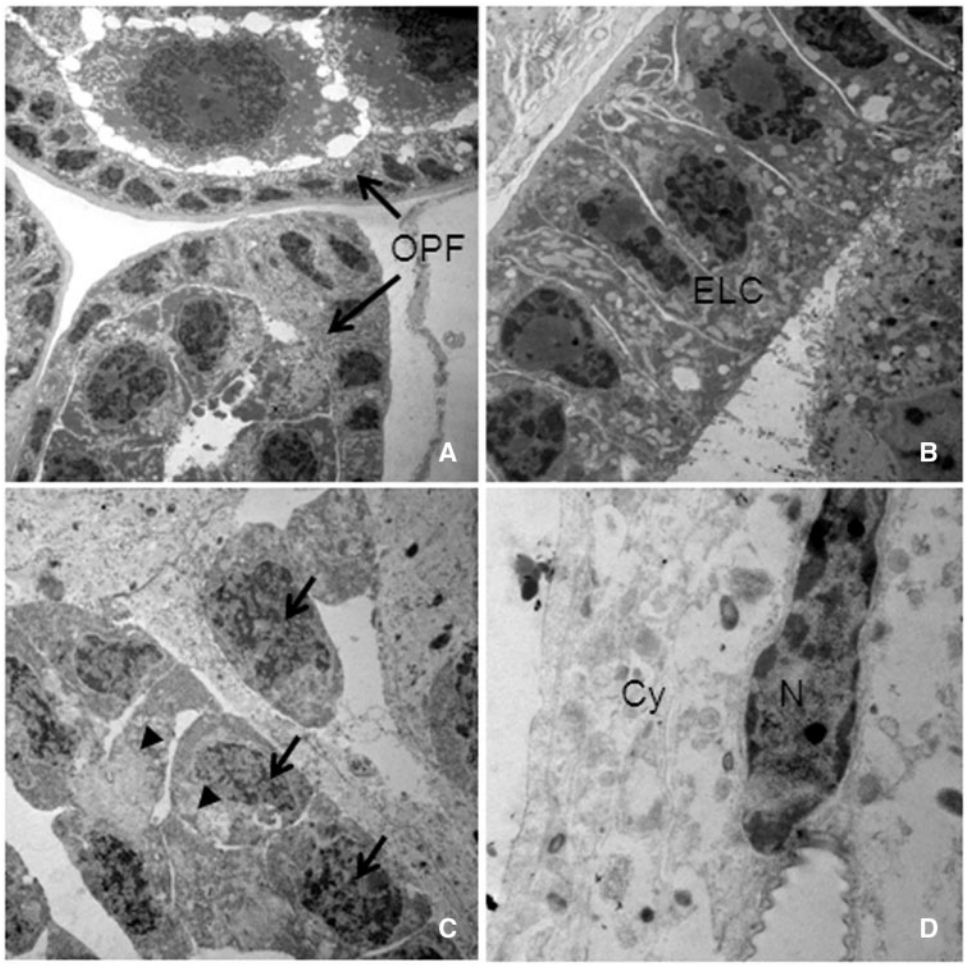

Fig. 3. Ultrastructure of ovaries from untreated (A, B) and thermically treated (C, D) adult *A. ludens* at 46°C for 12.9 min in vitro. (A) Morphology of primary follicles in ovary of control flies. (B) Higher amplification of external layer of cells. (C) Ovarian cells of primary follicles (OPF) showing hypertrophy mainly in the external layer of cells with alteration on cytoplasm (head arrows) and nucleus (arrows), which at higher amplification shows complete cytoplasm disintegration with irregular cytoskeleton (D). N, nucleus; ELC, external layer cells; Cy, cytoplasm. Amplifications: A, C = ×7,500; B = ×15,000; D = ×17,000).

The hydrothermal treatment using a temperature of 46.1°C for 90 min, plus 10 min if there is hydrocooling is a standard procedure to ensure the safety of round mango varieties ranging from 501 to 700 g, given that fly larvae are eliminated. The inconvenience of this method is that the quality and shelf life of the product is reduced.

In this work, cellular alterations were found in flies from third-instar larvae thermally treated for 12.9 min (70% mortality) and only 3.75% reached the adult stage. It is important to mention that one adult (11.1%) from the treated larvae showed malformations of the wings (Fig. 2B) and therefore an inability to fly. In the ultrastructural study

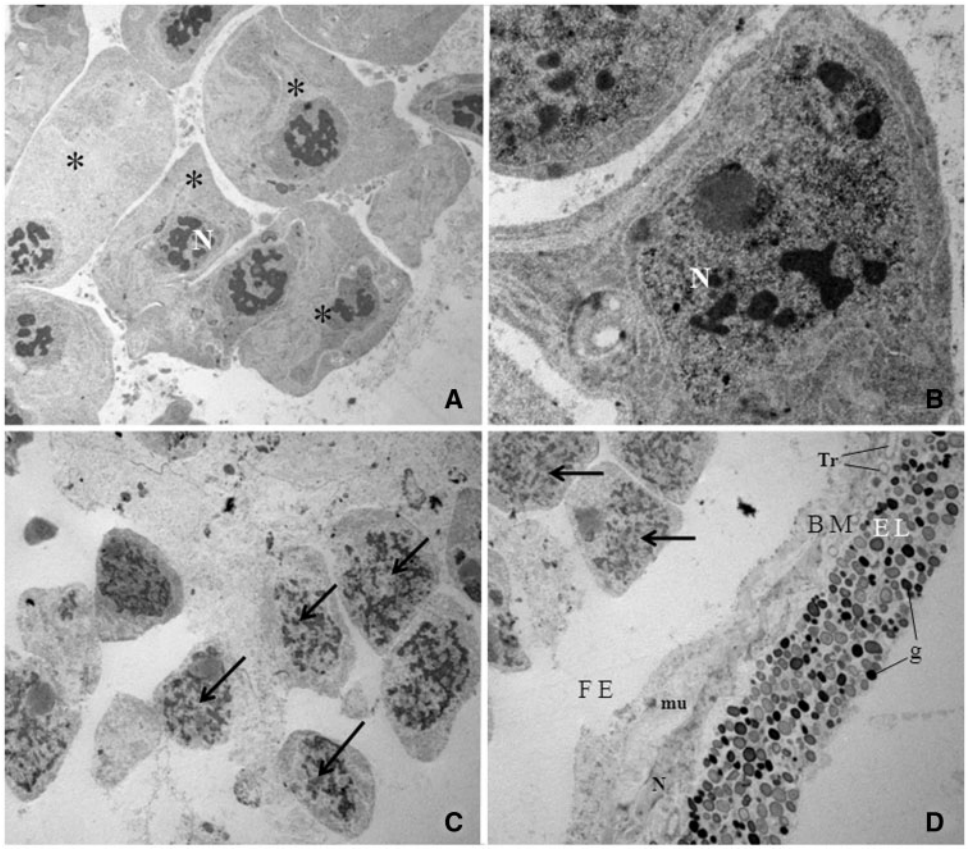

Fig. 4. Germinal cells from untreated and treated *A. ludens* testes. (A, B) Follicular cells of epoxy-resin-embedded testes from control fly showing normal cells (asterisks) with regular nucleus and cytoplasm. (C) Follicular cells of the germarium zone from testes isolated from treated specimen at 46°C for 12.9 min in vitro. Cellular damage and nuclear changes are shown with heterogeneous chromatin. (D) Longitudinal section of testes shows the external layer (EL) with clear damage on the basement membrane (BM) and nuclei (arrows) of cells in the germarium zone. FE, follicular epithelium; g, grains; mu, muscle; N, nucleus; Tr, tracheoles. Amplifications: A, C, D = ×7,500; B = ×13,000.

performed by transmission electron microscopy, all survivors showed alterations in the germinal cells.

A 94.6% mortality was observed after exposure to 46.1°C for 19.6 min, but no surviving larvae developed into adults. The results of the histological analysis showed that larvae underwent important cellular damage by the thermal treatment, which could explain why some treated larvae survived and only a small proportion (13 pupae in this study) developed into the puparium stage, but none of them reached the adult stage. Meanwhile, larvae exposed to the in vitro thermal treatment at the same temperature, but for 12.9 min, reached 70% mortality and the surviving larval cells also showed cellular damage, although in lower proportion compared with those from the 19.6 min treatment; this could explain the fact that 3.75% of these larvae successfully reached the adult stage.

Treated flies showed hypertrophy in cells of primary follicles, mainly in the external layer and in the basal region of ovaries. The morphology of testes from untreated flies was in accordance with a previous report of the ultrastructure of *A. ludens* testes (Valdez 2001). However, in treated male flies, the germarium zone showed an important cellular atrophy and follicular cell destruction was evident, mainly in the basement membrane and follicular epithelium of testes. Valdez (2001) reported that follicular epithelium seems to be related to germ-cell development.

These results show that using sublethal times (12.9 min and 19.6 min) at 46.1°C, cells of the treated larvae showed cytoskeletal alterations and damage of the nuclear membrane. However, only some larvae from the 12.9 min (70% mortality) developed into adults, showing ultrastructural changes in the sexual organs that suggest infertility, although additional studies are required to demonstrate this. These data may be useful to further investigate the molecular basis of survival of the insect upon a heat stress, as well as to design less drastic thermal treatments that preserve better the quality and shelf-life of the fruit.

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