ORGAN CULTURE OF SALIVARY GLANDS OF MALE *ANASTREPHA SUSPENSA* (DIPTERA:TEPHRITIDAE)

REJANE R. DE MORAES[1], JAMES L. NATION AND JAMES E. MARUNIAK
Department of Entomology and Nematology Hull Road, Building 970
University of Florida
Gainesville, FL 32611

[1]Fellow of National Research Council (CNPq). Brasilia, Brazil

ABSTRACT

Salivary glands from male Caribbean fruit flies, *Anastrepha suspensa,* were isolated individually by dissection and cultured in Schneider's medium containing 10% Fetal Bovine Serum and antibiotics. A total of 103 salivary glands were successfully cultured. Two different procedures were used to evaluate viability. A trypan blue dye test revealed that no more than 10% of the cultured cells were non-viable, since they acquired blue coloration. In the second set of experiments, acid phosphatase activity of the cultured glands was measured colorimetrically from absorbance at 415 nm of liberated p-nitrophenol. This method indicated that the cultured glands stayed metabolically active, with enzyme activity equal to that of freshly excised glands on the fifth, eighth, and fifteenth days of the experiment. Scanning electron microscopy (SEM) showed no visual differences in tissue organization and size of individual cells when freshly excised salivary glands and cultured glands were compared. SEM showed that the cells of cultured glands were neither swollen nor shrunken, and close-up views showed no evidence of cell deterioration or lysing.

Key Words: Salivary glands, organ culture, Caribbean fruit fly, Tephritidae, *Anastrepha suspensa,* acid phosphatase.

RESUMEN

Las glándulas salivares de los machos de la mosca caribeña de las frutas, *Anastrepha suspensa*, fueron aisladas individualmente por disección y cultivadas en medio de Schneider que conteniene 10% de suero fetal bovino y antibióticos. Un total de 103 glándulas salivares fueron cultivadas con éxito. Dos procesos diferentes fueron usados para evaluar la viabilidad. Una prueba de tinción de trypan azul reveló que menos del 10% de las células cultivadas no era viable porque adquirían una coloración azul. En una segunda serie de experimentos, la actividad de fosfatasa ácida de las glándulas cultivadas fue estudiada colorimétricamente mediante la absorbancia de p-nitrofenol liberado a 415 nm. Este método indicó que las glándulas cultivadas permanecían metabólicamente activas, con actividad enzimática en los días quinto, octavo y décimoquinto del experimento igual a la de las glándulas recién extirpadas. La Microscopía Electrónica de Barrido (MEB) no mostró diferencias visuales en la organización de los tejidos y en el tamaño de las céllulas individuales, cuando fueron comparadas las glándulas frescas y las cultivadas. La MEB mostró que las células de las glándulas cultivadas no estaban hinchadas ni contraídas, y las vistas tomadas de cerca no evidenciaron deterioración o rompimiento celular.

Organ culture is a technique in which whole organs or representative parts are maintained as tissues and retain their intrinsic distribution, numerical and spatial

orientation of explanted cells (Freshney 1987). Most organ cultures do not grow or, if they do, proliferation is limited to the outer cell layers. Thus, organ culture is essentially a technique for studying the behavior of integrated tissues rather than isolated cells.

The in vitro culture of insect organs has made much progress in the last 30 years. This success is due mainly to the development of adequate tissue culture media by scientists, e.g., Schneider (1964). Insect organ culture has been associated primarily with insect endocrinological studies (Marks 1976). In vitro cultures of imaginal discs of *Drosophila melanogaster* Meigen (Raikow & Fristrom 1971), *Sarcophaga peregrina* (Robineau-Desvoidy) (Ohmori & Ohtaki 1972), *Plodia interpunctella* (Hubner) (Oberlander 1976) and others have aided in the understanding of the role of 20-hydroxyecdysone and ecdysone in development and metabolic activity. Organ cultures of chironomid salivary glands have been extensively utilized in investigations of polytenic cell differentiation, cytogenetics, biochemistry and physiology (Firling & Kobilka 1978; Firling & Hou 1980).

The salivary glands in *Anastrepha suspensa* (Loew) are sexually dimorphic, with males having much enlarged glands that terminate on each side of the body in a large ball of convoluted tubules in the pleural region of abdominal segments 3, 4 and 5 (Nation 1974, 1981). The glands in males reach maximum size at approximately the same time that males are maximally active in sexual behavior and in producing a pheromone that attracts females (Nation 1972). Pheromonal components have been detected in dissected salivary glands by gas chromatographic analysis (Nation 1989), but conclusive evidence that the glands participate in pheromone production is lacking. Organ cultures of the salivary glands might be used to determine whether or not they are involved in pheromone production, if a culture technique for these glands was available. For example, Srinivasan et al. (1979) successfully cultured sex pheromone glands of *P. interpunctella* for 10 days and were able to recover sex pheromone from the culture medium.

The objectives of this study were to establish a protocol for sterile dissection of salivary glands from male Caribbean fruit flies; to maintain salivary glands in artificial medium for at least seven days; and to establish protocols for monitoring the viability of cultured glands.

Materials and Methods

Male *A. suspensa* were obtained from the mass rearing facility at the Florida State Department of Agriculture and Consumer Services, Gainesville, FL. Organ cultures of the male salivary glands were established in four separate experiments after dissecting the glands from 7- to 15-day-old males. All equipment and tools used to dissect the glands were sterilized. Microscopes, lamps and wax dishes were sterilized with 70% ethanol and left under a UV lamp for 20-30 min before dissections began. Scissors and forceps were autoclaved before each series of dissections and flamed after each fly was dissected. Glands were dissected under sterile conditions in a sterile hood. Male flies were surface-sterilized by immersion in 70% ethanol for 10 min and rinsed in sterile saline solution for 10 min. Just prior to each dissection, the head and legs of each fly were removed to diminish movement and to facilitate salivary gland removal. Each fly was pinned ventral side up in a wax dish containing sterile saline solution. A longitudinal cut was made through the cuticle and the lateral sides of the abdomen were gently pressed to release the salivary glands, which were then carefully excised. The excised glands were placed in sterile saline solution until a series of dissections were complete, then they were transferred to the culture medium.

Organ Culture

Schneider's medium (Schneider, 1964) containing 10% Fetal Bovine Serum and the antibiotics penicillin (100 units per ml), streptomycin (100 µg per ml), amphotericin B (0.2 µg per ml), gentamycin (50 µg per ml), geocillin (100 µg per ml), kanamycin (100 µg per ml) and tetracycline (10 µg per ml) were used to culture the salivary glands. [Schneider's medium has been used successfully to culture *Drosophila* cells (Schneider & Blumenthal, 1978).] A 24-cluster-well tissue culture plate (Corning) was used to culture the glands with 0.5 ml medium and two salivary glands per well. The salivary gland cultures were kept in an incubator at 27°C. Four experiments were conducted for eight to 15 days; the medium was replaced every three days in experiments 2 to 4.

Viability Tests

In the first experiment, penetration of trypan blue dye was used as an indicator of viability of the cultured glands, based on the principle that dead cells allow the dye to penetrate while live cells exclude the dye. Each day during one week of culture, 250 µl of trypan blue was added to the culture medium in one of the wells. Penetration or exclusion of the dye in the 2 glands was determined by microscopic examination. In experiments 2, 3 and 4, viability was monitored by determining the activity of acid phosphatase on the fifth, eighth and (only in experiment 4) on the fifteenth day. On each of these days, about 10 glands were removed from culture, rinsed with saline solution, and homogenized in 250 µl of 0.1 M citrate buffer, pH 5.5. Seventy µl of the glandular homogenate was added to each of two tubes containing 1 ml of 7 mM p-nitrophenol phosphate in 0.1 M citrate buffer, pH 5.5. A third tube served as a control and contained only citrate buffer. Fresh glands were dissected from flies that were the same age as the cultured glands, and these fresh glands were homogenized as described above for the cultured glands. Assay tubes identical to those for cultured glands were prepared to receive 70 µl of the fresh gland homogenate positive controls. All tubes were incubated at 30°C for 10 min. The reaction was stopped by adding 2 ml of 10% Na_2CO_3 to each tube. This altered the pH, stopped acid phosphatase activity and caused a shift in the electron structure of the released p-nitrophenol so that it gave a yellow color and absorbed light strongly at 415 nm. The amount of p-nitrophenol released was determined at 415 nm in a Spectronic 20D colorimeter. A standard curve was prepared from pure p-nitrophenol, and final results were expressed as pg p-nitrophenol released per gland per h.

The above protocol was changed slightly for the fourth experiment as follows. The substrate was dissolved in 0.1 M citrate buffer (pH 5.5) just before use. Incubation tubes contained 2 ml of 7 mM p-nitrophenol phosphate and 0.1 ml of glandular homogenate. Tubes were incubated for 20 minutes at 30°C. The reaction was stopped by adding 3 ml of 10% Na_2CO_3 solution. The control contained 7mM p-nitrophenol in citrate buffer.

Scanning Electron Microscopy

Freshly dissected and cultured salivary glands were fixed for about 24 h in Bouin's fixative, washed in 70% ethanol to remove excess picric acid, and dehydrated through an alcohol series to 100% ethanol. Glands were transferred from 100% ethanol to hexamethyldisilasane (HMDS) (Nation 1983), transferred once to fresh HMDS and air dried. The dried tissues were mounted on stubs, gold coated with a sputter-coater, and observed in a Hitachi S 570 instrument with filament voltage at 15 or 20 KV.

Results and Discussion

A total of 103 excised salivary glands from adult male flies were successfully cultured in isolated organ cultures for up to 1 week, and, in one experiment, up to 15 days. Scanning electron microscopy (SEM) showed no visual differences in tissue organization and size of individual cells between freshly excised salivary glands and glands cultured for 8 days (Fig. 1). Cells in the cultured glands did not shrink, collapse or lyse. Each of the bilateral salivary glands has the appearance of a cluster of grapes, and each "grape" is a cell enclosing a cavity, with cells interconnected by a central canal (Nation 1974). The SEM was useful in viewing the surface appearance of the gland cells and in ascertaining that the individual cells were not swelling, shrinking, or lysing.

Cultured glands excluded trypan blue dye, an indication that the cells of the glands were alive and apparently functioning normally. Some blue cells were observed on the periphery of the glands, but their percentage was never greater than 10%. In a preliminary experiment to measure acid phosphatase activity, we found that 57 pg of p-nitrophenol per gland per h was released by glands cultured for 5 days post-dissection, and 61 pg was released by fresh glands of comparable age to the cultures. After 8 days of culture, 56 pg per gland per h of p-nitrophenol was released by cultured glands and 55 pg per gland per h by fresh glands of comparable age. The control was changed to contain 7mM p-nitrophenol in citrate buffer following this preliminary experiment and subsequent results are shown in Table 1. Those data show that acid phosphatase activity of freshly excised glands and cultured glands did not differ in glands that were 5, 8 or 15 days old. The substrate, p-nitrophenol phosphate, added to the standard caused a slight yellow color, lowering the values in Table 1. However, these values more accurately represent the true enzyme activity than the preliminary data above.

The surface sterilization of adult flies, and the antibiotics in the culture medium were effective in maintaining sterility, since only one preparation showed evidence of contamination by becoming cloudy and discolored. Contamination also could be reduced by submerging adult flies in 70% ethanol. For example, Ohmori & Ohtaki (1972) surface-sterilized larvae of *S. peregrina* by submerging them in 70% ethanol for several min prior to isolating their wing discs for organ cultures, and Oberlander & Leach (1979) surfaced sterilized larvae of *P. interpunctella* (Hubner) by submerging them in 70% ethanol for 20 min prior to dissecting imaginal discs for organ cultures. Schneider's medium, which was developed for *Drosophila* imaginal disc cultures (Schneider 1972), was adequate for culturing the glands of male Caribbean fruit flies.

The exclusion of trypan blue and the activity of the enzyme, acid phosphatase, appear to be useful indicators of viability in salivary gland cultures. Enzyme activity and protein synthesis have often been used to measure the viability of cultured organs and cells. Firling & Kobilka (1978) measured the ability of cultured *Chironomus tentans* (Fabricius) salivary glands to incorporate ^{14}C-leucine into trichloroacetic acid (TCA) precipitable proteins. Acid phosphatase as well as amylase and protease activity were monitored in cultured salivary glands of *Calliphora* larvae (Price 1974). Organ cultures of *C. tentans* salivary glands were capable of excluding a solution of 0.12% trypan blue, while injured cells did not exclude the dye (Firling & Kobilka 1978). The few blue cells observed on the surface of the male salivary glands in the present experiments may well have been injured in the dissection or handling procedures, thus causing them to leak and allow uptake of trypan blue dye. Monitoring for trypan blue dye uptake is easier than measuring acid phosphatase activity and might be the method of choice in any future experiments with salivary glands from fruit flies.

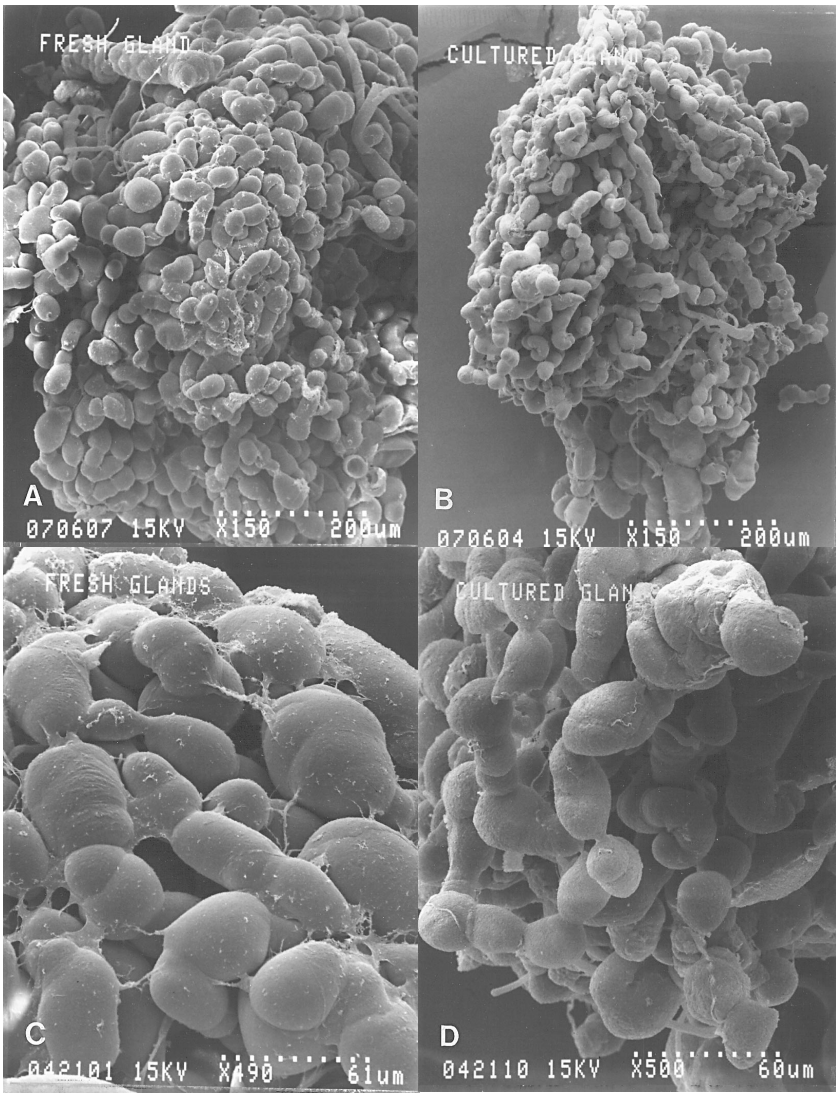

Figure 1. Scanning electron microscopy comparing cultured (8-days-old) and freshly excised male salivary glands (from 8-day-old males) from Caribbean fruit flies (*Anastrepha suspensa*). A and B, 150 × magnification; C and D, 500 × magnification.

ACKNOWLEDGMENTS

We thank Kathy Milne for technical assistance in the laboratory, Drs. Herbert Oberlander and S. M. Ferkovich for helpful criticism of earlier drafts, and the National Research Council-CNPq, Brazil, for financial support to R. R. de Moraes. Florida Agricultural Experiment Station Journal Series No. R-04079.

TABLE 1. COMPARISON OF PHOSPHATASE ACTIVITY BETWEEN CULTURED AND FRESHLY EXCISED SALIVARY GLANDS.

Salivary Glands	P-Nitrophenol pg per Gland per Hour		
	Mean[1] ± SD		
	5 Days	8 Days	15 Days
Cultured	7.36 ± 0.21	7.26 ± 0.23	6.69 ± 0.02
Fresh	8.38 ± 0.11	8.49 ± 1.57	7.32 ± 0.32

[1]All values are the mean of two replicates except at 8 days, when four replicates were analyzed.

REFERENCES CITED

FIRLING, C. E., AND B. KOBILKA. 1979. A medium for the maintenance of *Chironomus tentans* salivary glands in vitro. J. Insect Physiol. 25: 93-103.

FIRLING, C. E., AND A. HOU. 1980. Ecdysterone induced regression of *Chironomus* salivary glands maintained in vitro, p. 187-193 *in* E. Kurstak, K. Maramorosch, and A. Dubendorfer [eds.], Invertebrate systems in vitro. Elsevier/North Holland Biomedical Press, New York, NY. 598p.

FRESHNEY, R. I. 1987. Culture of animal cells: A manual of basic technique. 2nd ed. Alan R. Liss, Inc, New York, NY. 397pp.

MARKS, E. P. 1976. The uses of cell and organ cultures in insect endocrinology, *in* E. Kurstak, and K. Maramorosch, [eds.], Invertebrate tissue culture, applications in medicine, biology, and agriculture. Academic Press, New York. 398pp.

NATION, J. L. 1972. Courtship behavior and evidence for a sex attractant in the male Caribbean fruit fly, *Anastrepha suspensa*. Ann. Entomol. Soc. America. 65: 1364-1367.

NATION, J. L. 1974. The structure and development of two sex specific glands in male Caribbean fruit flies. Ann. Entomol. Soc. America. 67: 731-734.

NATION, J. L. 1981. Sex-specific glands in tephritid fruit flies of the genera *Anastrepha*, *Ceratitis*, *Dacus*, and *Rhagoletis* (Diptera: Tephritidae). Int. J. Insect Morphol. & Embryol. 10: 121-129.

NATION, J. L. 1983. A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. Stain Technology. 58:347-351.

NATION, J. L. 1989. The role of pheromones in the mating system of *Anastrepha* fruit flies, pp. 189-205 *in* A. S. Robinson and G. Hooper, [eds], Fruit Flies Their Biology, Natural Enemies and Control, Vol. 3A, Elsevier Press.

OBERLANDER, H. 1976. Hormonal control of growth and differentiation of insect tissues cultured in vitro. In Vitro. 12: 225-235.

OBERLANDER, H., AND C. E. LEACH. 1979. The action of β-ecdysone and juvenile hormone in organ cultures of lepidopteran imaginal disks. TCA Manual. 5: 993-995.

OHMORI, K., AND T. OHTAKI. 1973. Effects of ecdysone analogues on development and metabolic activity of wing disks of the fleshfly, *Sarcophaga peregrina*, in vitro. J. Insect Physiol. 19: 1199-1210.

PRICE, G. M. 1974. Protein Metabolism by the salivary glands and other organs of the larva of the blow fly, *Calliphora erythrocephala*. J. Insect Physiol. 20: 329-347.

RAIKOW, R., AND J. W. FRISTROM. 1971. Effects of β-ecdysone on RNA metabolism of imaginal disks of *Drosophila melanogaster*. J. Insect Physiol. 17: 1599-1614.

SCHNEIDER, I. 1964. Differentiation of larval *Drosophila* eye-antennal discs in vitro. J. Exp. Zool. 156: 91-104.

SCHNEIDER, I. 1972. Cell lines derived form late embryonic stages of *Drosophila melanogaster.* J. Embryol. Exp. Morphol. 27: 353-365.

SCHNEIDER, I., AND A. B. BLUMENTHAL. 1978. *Drosophila* cell and tissue culture, pp. 206-315 *in* M. Ashburner, and T. R. F. Wright, [eds], Genetics and Biology of *Drosophila*, Vol 2A, Academic Press, New York.

SRINIVASAN, A., J. A. COFELT, AND H. OBERLANDER. 1979. In vitro maintenance of the sex pheromone gland of the female indian meal moth *Plodia interpunctella* (Hubner). J. Chem. Ecol. 5: 653-661.