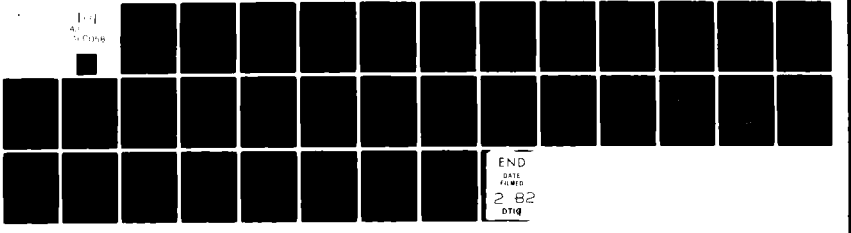
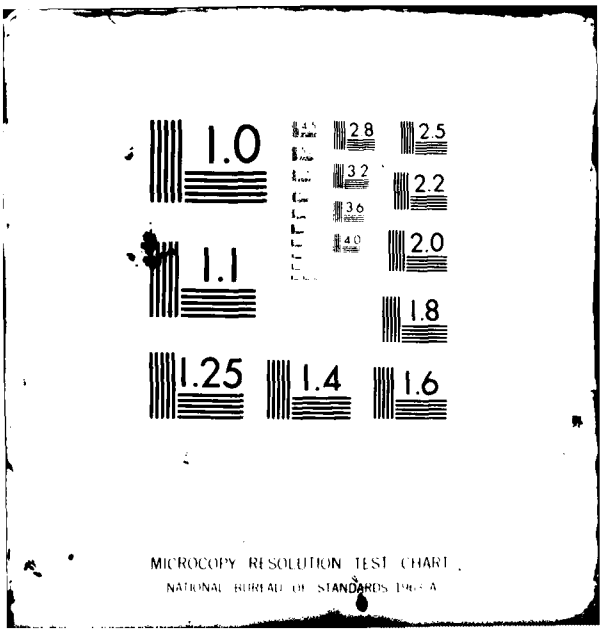
AD-A110 058 FLORIDA UNIV GAINESVILLE DEPT OF ENTOMOLOGY AND NEMA--ETC F/G 6/5
TRANSMISSION OF MICROSPORIDIAN PARASITES OF MOSQUITOES.(U)
JAN 82 D W HALL, E I HAZARD N00014-80-C-0172
UNCLASSIFIED NL



END
DATE FILMED
2 82
DTIC


1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS 1963-A

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: Annual Report No. 2	2. GOVT ACCESSION NO.: AD-A110 058
3. RECIPIENT'S CATALOG NUMBER:	
4. TITLE (and Subtitle): Transmission of Microsporidian Parasites of Mosquitoes	5. TYPE OF REPORT & PERIOD COVERED: Annual--Jan. 1, 1981 to Dec. 31, 1981
	6. PERFORMING ORG. REPORT NUMBER:
7. AUTHOR(s): Donald W. Hall and Edwin I. Hazard	8. CONTRACT OR GRANT NUMBER(s): N00014-80-C-0172
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Department of Entomology and Nematology, University of Florida, Gainesville, Florida 32611	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: NR 205 - 035
11. CONTROLLING OFFICE NAME AND ADDRESS: Office of Naval Research, Code 443, 800 N. Quincy St., Arlington, VA 22217	12. REPORT DATE: 1 January 1982
	13. NUMBER OF PAGES: 27
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office): SAME	15. SECURITY CLASS. (of this report): Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE:

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

SAME

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Mosquito, Aedes, Culex, Microsporidia, Amblyospora, vertical transmission, hybridoma, monoclonal antibody, ELISA

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A new microsporidian parasite of the mosquito *Aedes taeniorhynchus* is described and assigned to the genus *Microsporidium*. This parasite has a dimorphic developmental cycle and is transmitted transovarially from an infected female to her progeny.

Studies on the life cycle of *Amblyospora* sp. in *Culex salinarius* have shown that sporulation in the adult female mosquito is under host endocrine control. Sporulation can be initiated artificially by injection of ecdysone.

CONTINUED ON REVERSE SIDE

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE
S/N 0102-LF-014-6601

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. ABSTRACT--continued.

Techniques for purification of *Amblyospora* spores and preparation of spore antigens were developed, and mouse antibodies to these antigens were prepared. An enzyme-linked immunosorbent assay was developed for assay of candidate intermediate hosts for *Amblyospora* antigens. Preliminary work was done on development of monoclonal antibodies to *Amblyospora* antigens.

OFFICE OF NAVAL RESEARCH

Contract #N00014-80-C-0172

Task No. NR 205 - 035

Annual Technical Report Number 2

Transmission of Microsporidian Parasites of Mosquitoes

by

D.W. Hall
Department of Entomology and Nematology
University of Florida
Gainesville, Florida

and

E.I. Hazard
Gulf Coast Mosquito Research Laboratory
U.S. Department of Agriculture
Lake Charles, Louisiana

Microsporidia of the family Thelohaniidae are common parasites of mosquitoes and certain other invertebrates. They have complex life cycles and exhibit dimorphic development. In the adult female host the parasite forms small numbers of single binucleate spores which serve to infect the developing oocytes resulting in transovarial (vertical) transmission to the progeny of the infected female. Some species of microsporidia are transmitted in this manner for many generations while others are vertically transmitted for only 1 generation and all infected progeny die prior to reaching reproductive age. In both types of parasites a different type of spore is formed in the progeny than that formed in the infected female. These spores are uninucleate and packaged in groups of eight within a membrane. These uninucleate spores do not appear to be infectious when fed directly to mosquitoes.

We have shown that vertical transmission alone is not sufficient for maintenance of at least some of these parasites in nature. However, at the present time, none of these parasites have been successfully transmitted in the laboratory except by vertical transmission.

This contract is concerned with the microsporidian genera *Amblyospora* and *Parathelohania* and certain species of other genera which have dimorphic life cycles and are transovarially transmitted in mosquitoes. The primary objectives of this research are to work out the life cycles of selected parasites and to determine the mechanism of horizontal transmission of the parasites from mosquito to mosquito.

This research was supported in part by the Office of Naval Research, Microbiology Program, Naval Biology Project, under Contract #N00014-80-C-0172, NR 205 - 035.

Life Cycle Studies.

Due to the extreme drought conditions in Florida this year, we were unable to collect some of the desired mosquito species in sufficient numbers for life cycle studies of their microsporidian parasites. Consequently, work was concentrated on a new microsporidian species from the black salt-marsh mosquito *Aedes taeniorhynchus* which we are naming *Microsporidium fimbriatum* and *Amblyospora* sp. from *Culex salinarius*. A short description of *M. fimbriatum* follows.

Microsporidium fimbriatum.

In the course of screening the progeny of female *Aedes taeniorhynchus* for transovarially transmitted pathogens egg batches infected with a microsporidium which resembles none previously described from mosquitoes were found.

Egg batches were obtained from individual mosquitoes collected in Everglades National Park by giving them a blood meal and placing them in vials containing gauze moistened with .15% NaCl. The resultant eggs were hatched and reared to screen for parasites. Screening involved examination of fourth instar larvae against a black background to detect discoloration and squashing emerged adults for examination with phase contrast microscopy. Some of the individuals from infected egg batches were smeared and stained with Giemsa, while others were prepared for electron microscopy.

Some eggs from 2 batches were infected with the microsporidium. In the first of these, 6 larvae developed patent infections in the terminal abdominal segments, most obviously in the fifth segment. One of these larvae was triturated and fed to 24 hr. old *A. taeniorhynchus* larvae which

showed no patent infection when reared to the fourth instar, indicating a lack of *per os* transmission. The remainder of the larvae from the infected egg batch were reared to the adult stage and examined for infection. Of the 16 adults that emerged, 12 females showed no sign of infection, but all 4 males contained numerous spores.

The second infected egg batch was hatched in two lots. The first lot was reared to the adult stage and found to contain one infected male. Smears were made with the female that laid the eggs and with the individuals from the second lot at intervals through their development and were stained with Giemsa. Three of these smeared individuals, a third instar larva, a fourth instar larva, and a pupa, were found to harbor the parasite. The smear of the parent female contained diplokaryotic stages of the parasite and empty cylindrical spore walls (Fig. 1) which appeared similar to the walls of the spores which function in transovarial transmission in members of the family Amblyosporidae (Hazard and Weiser, 1968; Andreadis, 1978).

The third instar larva contained many diplokaryotic stages (Fig. 2,9) which are apparently the meronts (the primary multiplicative stages). They also contained a smaller number of binucleate and tetranucleate sporonts (Fig. 3), as well as a few scattered sporonts with six or eight nuclei (Fig. 4), sporoblasts (Fig. 5) and spores (Fig. 6,8). Sporogony continues through the fourth instar and pupal stage. Multinucleate sporonts appear to produce uninucleate sporoblasts by budding. There is apparently no pansporoblastic membrane, but this cannot be positively determined until it is possible to observe sporonts in electron micrographs.

Spores from larvae and adult males appear short pyriform with a sharp point at the anterior end when viewed in fresh smears. However, the point

is difficult to demonstrate in Giemsa stained smears and electron micrographs (Fig. 5-8). The exospore is characterized by the presence of a fine, dense fringe.

Because insufficient information on its development and morphology make its taxonomic position unclear, we have assigned the species to the collective genus *Microsporidium* suggested by Sprague (1977). Its development is most similar to dimorphic members of the family Amblyosporidae (Weiser, 1977) but the form of the spores found in males and the question of the pansporoblastic membrane preclude placement in this family at this time. We also do not know whether or not there is meiosis in the developmental sequence of the spores as has been reported for certain other microsporidia with dimorphic development (Hazard et al., 1979). The specific epithet, *fimbriatum*, means "fringed" in reference to the exospore of spores in male larvae.

Microsporidium fimbriatum sp.n.

Host. The black saltmarsh mosquito *Aedes taeniorhynchus* (Wiedemann).

Type locality. Coastal Prairie Trail, Everglades National Park, Florida.

Vegetative stages. Meronts have one or two diplokarya.

Sporulation stages. Sporonts found in third and fourth instar larvae and pupae contain two, four, six, or eight nuclei. No pansporoblastic membrane is evident in the light microscope.

Spores. Two types of spores are produced, cylindrical spores in the adult female and uninucleate, short pyriform spores, having fringed exospores, in the last instar larvae, pupae, and adult males.

Type material. Holotype slides will be sent to the United States National Museum.

Amblyospora sp.

The general life cycle and quantitative aspects of vertical transmission of *Amblyospora* sp. in *Culex salinarius* has been worked out previously (Andreadis and Hall, 1979a, 1979b). However, there are still certain questions remaining regarding events in the adult female mosquito which lead to vertical transmission. Although there is a high efficiency of vertical transmission to progeny of infected females, over at least 5 gonotrophic cycles large numbers of infected oenocytes are never seen in Giemsa-stained smears of these mosquitoes. In an attempt to solve this puzzle, infected adult females of different ages were serially sectioned, and the total infected oenocytes were counted. The results for nulliparous females are given in Table 1. Results for subsequent ovarian cycles are not yet tabulated. The average number of oenocytes per female for young females is 35.4. The number of parasites per oenocyte is variable but a realistic average might be 100. Based on an average of 327 eggs per female over 5 gonotrophic cycles, this provides for a potential multiplicity of infection of 11. An infected oenocyte is shown in Figure 10. It is evident that the oenocytes harbor a sufficient quantity of parasites to account for the observed rate of vertical transmission.

An interesting observation from the histological sections was that the infected oenocytes were somewhat randomly distributed throughout the bodies of the mosquitoes with a few even being found in the heads. Healthy larval oenocytes normally degenerate during the pupal stage and are restricted to the abdomen whereas infected ones persist in the adult stage and are freed to circulate in the hemocoel. Since the infected oenocytes are rarely adjacent to the ovaries, it is obvious that penetration of the

ovaries by the polar filaments (the normal infection mechanism in Microsporidia) is unlikely. It is probable that the sporoplasms are released through the polar filaments into the hemolymph and then carried to the ovaries where penetration occurs by an as yet unknown mechanism.

Another intriquing problem in the life cycle of *Amblyospora* sp. was the mechanism controlling sporulation in the adult female. The spores are formed synchronously with development of the ovarian follicles following a blood meal which is known to trigger the hormonal sequence leading to egg development. This suggests that the parasite may either respond to the nutrients released into the hemocoel from the blood meal or that there may be a more intimate host-parasite relationship in which the microsporidium responds to the physiological changes associated with the host's gonadotrophic cycle. In addition to blood meal nutrients the substances known to appear in the hemolymph during this cycle are vitellogenins and hormones, particularly egg development neurosecretory hormone (EDNH) (Lea, 1972) and ecdysteroids secreted by ovaries (Hagedorn et al., 1975). A study was conducted to determine which, if any, of these factors is used by *Amblyospora* sp. as a cue to initiate sporulation.

Treatments.

Healthy and *Amblyospora* infected adult female *C. salinarius* from laboratory colonies were used for experiments 5 and 6 days post-emergence. For all treatments mosquitoes were lightly anesthetized with nitrogen gas. Injections and topical applications were done with a finely drawn capillary tube calibrated to approximately 1 µl. Cholesterol and 20-hydroxyecdysone were dissolved in insect saline with 10% ethanol. Methoprene and juvenile

hormone (JH) I were dissolved in acetone. Egg macerate was prepared by removal of ovaries from gravid mosquitoes, maceration in a ground glass tissue grinder with 0.15 M NaCl buffered to pH 6.9, and centrifugation at ca. 800 g to remove particulate material.

Surgery.

To remove the medial neurosecretory cells and corpora cardiaca, the sources of EDNH, mosquitoes were decapitated, and the wound was sealed with paraffin. Decapitations of blood engorged individuals were done within 30 min. of feeding.

Ovariectomies were performed on mosquitoes starved for 24 hrs. prior to surgery. After placing them in a mold of modeling clay with thin strips of clay across the thoraxes as restrainers, the abdomens were immersed in saline. Sharpened jeweler's forceps were then used to remove the ovaries through single ventral incisions between the 6th and 7th abdominal sternites. Wounds were sealed with paraffin. Operated individuals were offered a guinea pig blood source after 6 hrs., then placed in humidified chambers.

Scoring.

Unless otherwise indicated all individuals were scored 48 hr. after treatment. Blood feeding of infected mosquitoes results in completion of sporulation after that length of time. Mature spores are then detectible for another 12-24 hr. (Andreadis and Hall, 1979a, 1979b).

Slides were prepared for screening for sporulation by smearing whole mosquitoes and staining with Giemsa stain after methanol fixation. Slides with a single spore were scored as positive. Those with at least 30 veg-

etative *Amblyospora* and no spores were scored as negative for sporulation. Spores and vegetative stages were easily distinguishable in Giemsa preparations (Fig. 10,11).

Results.

Decapitation of host mosquitoes immediately after blood feeding prevented the sporulation of *Amblyospora* that otherwise followed the blood meal (Table 2). This indicates that factors other than nutrients from digested blood and gut stretch stimulus trigger the spore formation process. The presence of spores in 2 of the decapitated controls which were not given blood is probably due to autogeny which is present in a small percentage of the mosquitoes in our colony.

Since vitellogen appears in mosquito hemolymph shortly after a blood meal this protein must be considered as a candidate for the sporulation cue for *Amblyospora*. The vitellin of mature mosquito eggs is immunologically indistinguishable from its vitellogenin precursor (Hagedorn et al., 1978). With this in mind and with the consideration that other egg substances are presumably among those that appear in the hemolymph during gonadotrophic development it was decided to use the soluble material from a macerate of mature eggs for injection into infected *C. salinarius*. A 2-fold dilution of a concentration that proved lethal was used. In only 1 of 20 treated individuals were spores found (Table 3). Again the occurrence of autogeny may account for the presence of spores. Egg proteins appear not to induce sporulation of *Amblyospora*.

The approach taken to the question of hormonal induction of sporulation was to treat infected mosquitoes with the hormones and their analogs. Since EDNH is not commercially available, indeed its exact nature is unknown, this

hormone could not be used.

JH is required for the development of mosquito ovaries to the previtellogenic stage (Lea, 1963, 1969). Borovski (1981) has speculated on a vitellogenic role for this hormone. To determine if it has an effect on *Amblyospora*, JH I and its more stable analog methoprene were applied topically to infected *C. salinarius*. JH I was ineffective a 1 µg, and methoprene was ineffective up to a dose of 6 µg which was lethal to most mosquitoes (Table 4).

Shortly after a mosquito takes a blood meal its ovaries secrete ecdysone, which is then hydroxylated by other tissues to form the more active 20-hydroxyecdysone. This hormone, when injected into infected mosquitoes at low doses, did not induce sporulation. At a dose of 2.5 µg all 32 of the individuals injected contained spores (Table 5). This is a pharmacological rather than a physiological dose. The fact that such a large dose is required may be explained, at least in part, by the rapid degradation of ecdysteroid in vivo (Ohtaki and Williams, 1970). It seems likely from these results that 20-hydroxyecdysone, or some substance whose presence is induced by it, is the cue to which *Amblyospora* responds by sporulating.

To see if this was a general response to steroids cholesterol was injected into infected *C. salinarius*. This was without effect (Table 6).

Since the ovaries are the source of ecdysteroids in blood-fed mosquitoes we decided to determine whether *Amblyospora* would sporulate in mosquitoes which were given blood after the removal of their ovaries. In none of those so treated did sporulation occur (Table 7). 20-hydroxyecdysone induces synthesis of dopa decarboxylase in insects (Karminsky et al., 1980). This enzyme catalyzes production of catecholamines involved in sclerotization of

the insect cuticle and presumably the egg chorion. Furthermore the microsporidian spore wall resembles to cuticle in being of protein and chitin (Vaura, 1976). To see if catecholamines appearing in the hemolymph following 20-hydroxyecdysone are a sporulation trigger, dopamine and N-acetyldopamine were injected into the host. Neither was effective (Table 8).

Finally, to determine if the spores formed in response to 20-hydroxyecdysone are viable, electron micrographs were prepared from hormone injected and blood-fed mosquitoes. There were no discernible differences in the ultrastructure of the resultant sporoblasts.

In summary it is concluded that 20-hydroxyecdysone induces sporulation of *Amblyospora* in *C. salinarius*. Whether the parasite responds directly to the hormone or to some substance that appears in the hemolymph as a result of the hormone's presence has yet to be determined.

A proposed scheme for sporulation and subsequent infection of the developing eggs is presented in Figure 12.

Horizontal Transmission Studies.

Since the haploid spores formed in large numbers in male *C. salinarius* larvae do not appear to be infective when fed back to healthy larvae, we have hypothesized the existence of an intermediate host. Several approaches are possible to test this hypothesis. The first is to feed the spores to candidate intermediate hosts and look for signs of development of the parasite. It is also possible that the spores may be conditioned by passing through the digestive tracts of other organisms. Very preliminary studies to investigate the first possibility have been done and will be expanded next year.

A third approach which is being investigated is the use of highly specific hybridoma monoclonal antibodies to attempt to detect *Amblyospora* antigens in candidate intermediate hosts by utilizing the highly sensitive enzyme-linked immunosorbent assay (ELISA). The background work in developing the methodology for this approach was done this year.

Methodology.

Spore purification.

A method for purification of *Amblyospora* haploid spores for antibody production was developed. Prior to purification, spores were stored at -80°C. Freezing appears to prevent extrusion of the polar filament during purification. Infected larvae are macerated in phosphate buffered saline (PBS) in a tissue grinder and then subjected to 2 cycles of differential centrifugation. The spores are then layered onto 0-10% Ludox HS-40 density-gradients and centrifuged for 4 hours at 1500 g. After washing with PBS 3 times to remove the Ludox, spores were stored at -80°C until needed.

A protocol was developed for the Enzyme-linked immunosorbent assay (ELISA) which is the assay to be used for possible detection of *Amblyospora* antigens in putative intermediate hosts. Known reagents (bovine serum albumin [BSA] and rabbit anti-BSA antiserum) were used as test reagents. Clean controls and sensitivities of 10^{-2} µg BSA were achieved.

Antigen Preparation.

Spores of *Amblyospora* sp. were stored for one month at -80°C, thawed, and homogenized in a Braun Tissue Homogenizer. The homogenate was dialyzed against several changes of PBS pH 7.4 at 4°C.

Immunization.

Balb-c mice were injected intraperitoneally with 0.35 ml of an emulsion made up of equal parts of Freund's Complete Adjuvant and the homogenate from $5x10^6$ spores. Two weeks later they were boosted intraperitoneally with 0.35 ml of homogenate from 10^7 spores without adjuvant.

One week after the second injection the mice were exsanguinated from the heart. The blood was allowed to clot for one hour at room temperature and overnight at 4°C and then centrifuged for 20 minutes at 3500 g. After decanting, the serum was heat inactivated for 30 minutes at 56°C. Immunoglobulin was precipitated with saturated ammonium sulfate and dialyzed against several changes of PBS pH 7.4. The Ab titer was not sufficiently high, and the mice were given an additional injection. The mice were then bled a month later. The serum was treated as above and run against soluble spore proteins in the ELISA assay. A sensitivity of 10 ng was achieved.

Hybridoma Technique.

The production of hybridoma antibody can be broken down into four basic procedures: growing and maintaining lymphocytes in cell culture, freezing them for long term culture followed by thawing and initiation of new cultures, cloning them, and fusing myeloma and spleen lymphocytes to form hybridomas. All of these procedures have now been performed.

Growth and Maintenance of Cells in Culture.

A T-75 flask of SP2/0 mouse myeloma cells was provided by Dr. Paul Klein of the Department of Pathology, University of Florida College of Medicine. The cells were maintained in a Napco model 5100 carbon dioxide incubator at 37°C and 6 to 7% CO_2. In a preliminary experiment growth rates

were compared on two media, Eagle Modified Minimum Essential Medium, "Auto-Pow" (Flow Laboratories, McLean, Va.) with supplements of L-glutamine, non-essential amino acids, pen-strep, and sodium bicarbonate, and Dulbecco's Modified Eagle Medium (Gibco Laboratories, Grand Island, N.Y.). The Dulbecco's proved superior and was selected for all subsequent work.

The cells show characteristic exponential growth (Fig. 1). By this time three dozen cultures have been set up in T-75 flasks, 24-well culture plates, and 96-well culture plates. Most of these were repeatedly sampled for cell counting and some were sub-cultured; all transfers were done in an Environmental Air laminar flow hood. Despite the problem of air-borne fungi which is well known to all who try to culture cells in Florida's hot and humid climate, only one case of contamination has occurred. This involved the original flask, which had been harvested and received fresh medium to see whether any cells could be recovered.

Freezing and Thawing Cells.

The freezing and thawing protocol followed was that of Kennett (1980a). Cells were centrifuged for 5 minutes at 1500 RPM and resuspended at 10^7/ml in 1.0 ml aliquots of ice-cold 5% dimethylsulfoxide (DMSO) in newborn calf serum in 2.0 ml Nunc vials; these were placed immediately in the Revco (-70°C). After several weeks in storage cells were thawed rapidly by immersing the vials in a 37°C water bath. Warming of the medium was prevented by removing vials from the bath before the aliquot had thoroughly melted and allowing the melting center core to cool the medium. One ml of chilled culture medium was added to the vial over the course of 30 sec and the contents then transferred to 10 ml of medium so as to reduce DMSO withdrawal

shock (George Guttman, pers. comm.). The cells were centrifuged in a refrigerated centrifuge for 5 min at 1500 RPM and then diluted four-fold in medium in a T-75 flask. After 24 h the cells were counted and their viability was checked using 0.1% aqueous trypan blue (N. Das and S. Zam, pers. commun.). Cells frozen and thawed with this protocol had a viability between 10 and 16%. We are experimenting with a different freezing medium (10% DMSO, 30% serum, and 60% culture medium, P. Klein, pers. commun.) and two different freezing protocols (in vapor phase in the liquid nitrogen refrigerator using the Linde Biological Freezing Unit, and in the Revco using paper towel wrapping of the vials to reduce the freezing rate, (G. Guttman, pers. commun.) to determine the best way to improve viability of the cells.

Cloning.

Cells were cloned using the method of limiting dilution (McKearn, 1980), using conditioned medium in place of thymocyte "feeder cells" (N. Das and P. Zam, pers. commun.). SP2/0 cell suspensions were prepared in 50/50 conditioned/fresh Dulbecco's medium at 500, 50, and 5 cells/ml. 0.2 ml of each suspension per well was plated out in 96-well tissue culture plates. The percent of positive wells for each suspension in four cloning experiments is shown in Table 9. The rule of thumb is that suspensions producing less than 30% positive wells have probably been cloned successfully (McKearn, 1980); this criterion was met by two of the 5 cell/ml suspensions. Microscopic examination confirmed that positive wells in plates having fewer than 30% tended to represent single foci of cell growth and hence descent from single cells (see Fig. 1), whereas at least some wells in plates

having more than 30% positives had multiple foci of growth. After 14 days of growth macroscopic examination of plates shows positives as yellow due to alteration of pH by the growing cells, while negative wells remain red.

The results are entirely satisfactory. Nevertheless an experiment is being started to compare cloning efficiency with conditioned medium and thymocyte feeder cells from 6-week old Balb/c mice (Jackson Laboratories, Bar Harbor, Me.) to see if anything can be gained by the more elaborate procedure.

Fusion of Myeloma and Spleen Lymphocytes

Fusion was performed using Kennett's (1980b) protocol, with various modifications (N. Das, G. Guttman, P. Klein, and S. Zam, pers. commun.). A 15-week old Balb/c mouse was killed and aseptically splenectomized. The spleen was macerated by crushing with a lucite bar in 10 ml of serum-free Dulbecco's medium and drawing the pieces up into a Pasteur pipette. The suspension was allowed to stand for 10 min so that large pieces could settle and the single cell suspension drawn off, centrifuged at 1500 RPM for 5 min, and resuspended in 5 ml fresh serum-free medium. These cells were counted and the suspension adjusted to 2×10^7 ml.

Cells from an SP2/0 culture were centrifuged for 5 min at 1500 RPM, washed once in serum-free medium, and resuspended to 4×10^6/ml in 5 ml serum-free medium. The spleen and myeloma suspensions were combined in a round-bottomed 15 ml tube and centrifuged at 600 RPM for 12 min. The supernatant was carefully pipetted off and, over the course of 60 sec, 1.0 ml of 35% polyethylene glycol in serum-free medium at 37°C was pipetted onto the cell pellet and mixed with it by tapping. During the next 60 sec 1.0 ml of 37°C

serum-free medium was added, and during the next 4 min the cells were transferred to 20 ml of serum-free medium. The cells were then pelletted by centrifugation at 1500 RPM for 5 min and resuspended in two 50 ml tubes containing HT medium (Littlefield, 1964). These were left at room temperature for 12 h and the medium made selective (Littlefield, 1964) by the addition of 5×10^{-5} M amethopterin. The suspensions were then plated out into 6 96-well culture plates at 0.2 ml/well.

These cultures were fed on days 2, 4, and 7 by removing half of the medium and replacing it with HAT medium (Littlefield, 1964) on day 2 and HT medium on the other days.

The first hybridomas are now being prepared with cells from immunized mice using this same technique.

Collection of Candidate Intermediate Hosts for Serological Testing.

Large numbers of aquatic invertebrates representing many taxa have been collected, sorted, and stored at -80°C for future serological screening for *Amblyospora* antigens with hybridoma antibodies (Table 9).

TABLE 1. TOTAL OENOCYTE COUNTS FROM *Amblyospora*-INFECTED NULLIPAROUS ADULT FEMALE *Culex salinarius**

4-7 days old (non-blood-fed)	>10 days old (non-blood-fed)	21 days old (77 hr. post-blood-feeding)
50	39	29
33	23	30
27	24	21
22	32	16
12	11	37
42	13	18
56	31	10
41	44	30
	28	28
	50	

*Age refers to time since adult eclosion.

TABLE 2. EFFECT OF DECAPITATION OF NEWLY BLOOD-FED *C. salinarius* ON SPORULATION OF *Amblyospora* sp.

	Vegetative stages only	Spores
Blood-fed, decapitated	24	0
Blood-fed, not decapitated	0	20
No blood, decapitated	14	2

TABLE 3. EFFECT ON SPORULATION OF *Amblyospora* sp. OF INJECTION OF HOST EGG MACERATE INTO *C. salinarius*

	Vegetative stages only	Spores
Egg macerate	19	1
Saline	16	0

TABLE 4. EFFECT ON SPORULATION OF *Amblyospora* sp. OF TOPICAL APPLICATION OF JH I AND METHOPRENE TO *C. salinarius*

	Vegetative stages only	Spores
10 ng methoprene	20	0
60 ng methoprene	30 (5 at 72 hr)	0
1 µg methoprene	18	0
6 µg methoprene	12 (5 at 72 hr)	0
1 µg JH I	32	0
Acetone	14	0

TABLE 5. EFFECT ON SPORULATION OF *Amblyospora* sp. OF INJECTION OF 20-HYDROXYECDYSONE INTO *C. salinarius*

	Vegetative stages only	Spores
100 pg + 250 pg at 15 hr	8	0
10 ng	14	0
2.5 µg	0	32
Saline	28	0

TABLE 6. EFFECT ON SPORULATION OF *Amblyospora* sp. OF INJECTION OF CHOLESTEROL INTO *C. salinarius*

	Vegetative stages only	Spores
2.5 µg Cholesterol	16	0
Saline	12	0

TABLE 7. EFFECT ON SPORULATION OF *Amblyospora* sp. OF OVARIECTOMY OF *C. salinarius*

	Vegetative stages only	Spores
Ovariectomized and blood-fed	26	0
Sham-operated and blood-fed	1	16

TABLE 8. EFFECT ON SPORULATION OF *Amblyospora* sp. OF INJECTION OF CATECHOLAMINES INTO *C. salinarius*

	Vegetative stages only	Spores
10 µg Dopamine	16	0
1.5 µg N-acetyldopamine	23	2
Saline	20	0

TABLE 9. THE RESULTS OF FOUR CLONING EXPERIMENTS WITH SP2/0 CELLS. NUMBERS ARE PERCENT OF POSITIVE WELLS

	DILUTION		
Experiment	500 cells/ml	50 cells/ml	5 cells/ml
1	100	100	61
2	100	99	27
3	100	100	24
4	100	100	35

TABLE 10. TAXONOMIC AFFINITIES OF POTENTIAL INTERMEDIATE HOSTS COLLECTED AT SEVEN GAINESVILLE, FLORIDA SITES DURING 1981**

Phylum	Class	Order	Family
PLATYHELMINTHES	Turbellaria	Tricladida	Planariidae
ROTATORIA	Monogonta	Ploima	
ANNELIDA	Oligochaeta	Haplotaxida	Naididae
	Hirudinea		
ARTHROPODA	Crustacea	Ostracoda	
		Cladocera	
		Copepoda	
		Isopoda	
		Aphipoda	
		Decapoda	
	Arachnida	Hydrachnellae	
		Araneae	Lycosidae
			Pisauridae
	Insecta	Ephemeroptera	
		Odonata	
		Hemiptera	Notonectidae
			Naucoridae
			Nepidae
			Belostomatidae
			Hydrometridae
			Veliidae
		Neuroptera	Corydalidae
		Coleoptera	Gryinidae
			Hydrophilidae
			Dytiscidae
			Helodidae
		Diptera	Tipulidae
			Culicidae
			Chironomidae
			Stratiomyidae
			Ephydridae
			Ceratopogonidae
MOLLUSCA	Gastropoda		
	Pelecypoda		
CHORDATA	Pices	Osteichthes	
	Amphibia	Anura	

**Note: This list is conservative because many families are represented by more than one species.

Fig. 1. Empty spore case from adult female mosquito. Giemsa stain. X2,400.

Fig. 2. Diplokaryotic meront from male larva. Giemsa stain. X2,400.

Fig. 3. Tetranucleate sporont from male larva. Giemsa stain. X2,400.

Fig. 4. Octonucleate sporont from male larva. Giemsa stain. X2,400.

Fig. 5. Sporoblasts from male larva. Giemsa stain. X2,800.

Fig. 6. Mature spores from adult male. Giemsa stain. X2,300.

Fig. 7. Immature spore from adult male. EX, exospore; N. nucleus; P, polaroplast. X26,000.

Fig. 8. Mature spore from adult male. X14,000.

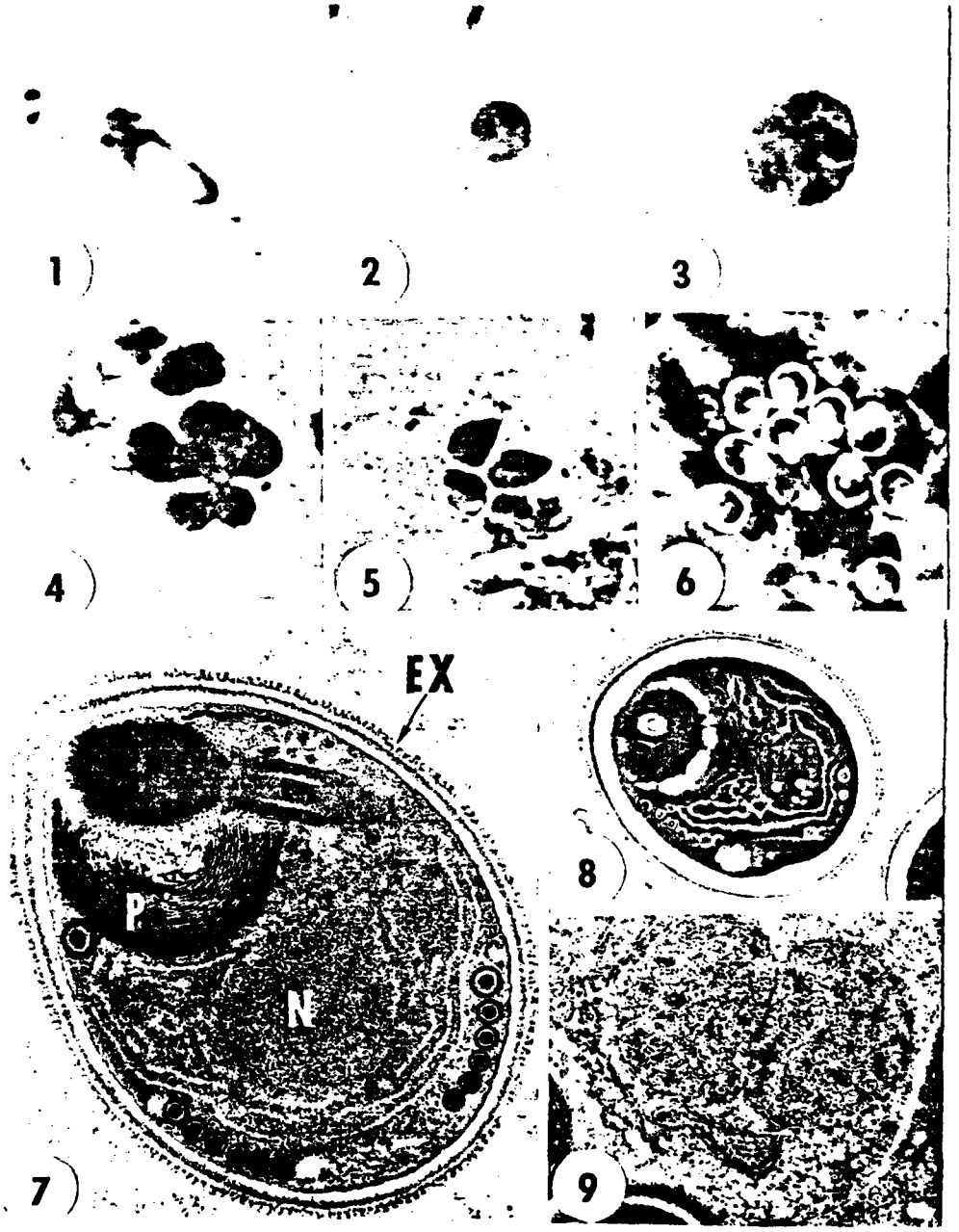
Fig. 9. Diplokaryotic stage. X15,000.

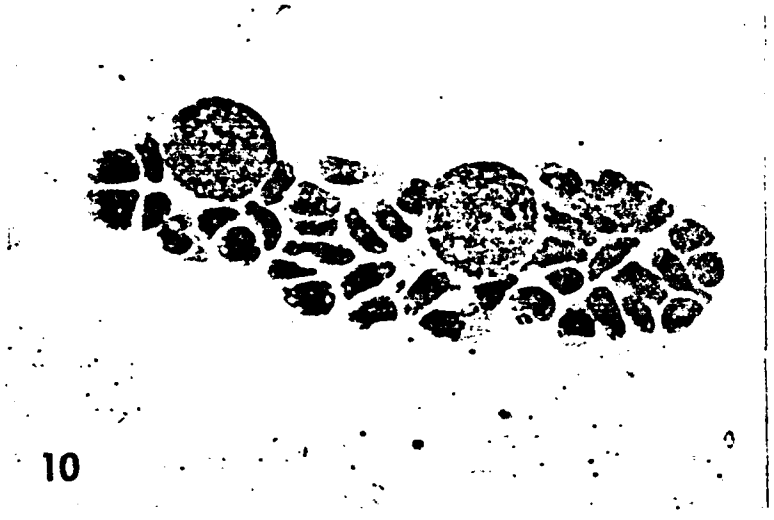
Fig. 10. Giemsa-stained oenocyte from *Amblyospora* infected adult female *Culex salinarius* with vegetative stages. X520.

Fig. 11. Giemsa-stained *Amblyospora* spores from adult female *Culex salinarius*. X630.

Fig. 12. Proposed scheme for sporulation of *Amblyospora* in female *Culex salinarius* and subsequent infection of developing eggs.

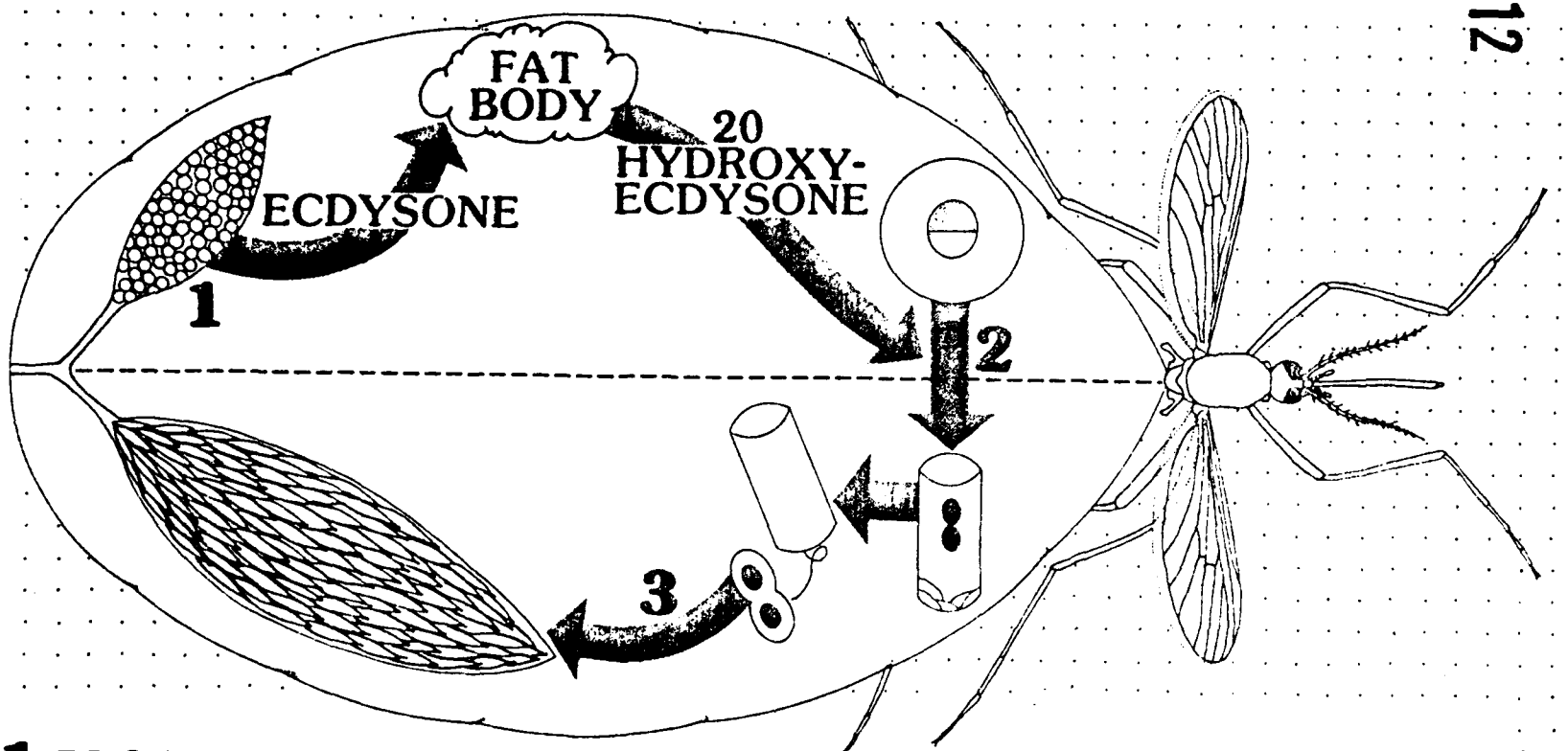
Fig. 14. Lymphocyte clone showing a single focus of growth.


1
2
3
4
5
6
EX
P
N
7
8
9



10

11



1 BLOOD MEAL TRIGGERS OVARIAN SECRETION OF ECDYSONE

2 SPORULATION IS COMPLETED BY 48 HR. POST BLOOD MEAL IN RESPONSE TO 20 HYDROXYECDYSONE

3 SPOROPLASM INFECTS DEVELOPING EGGS

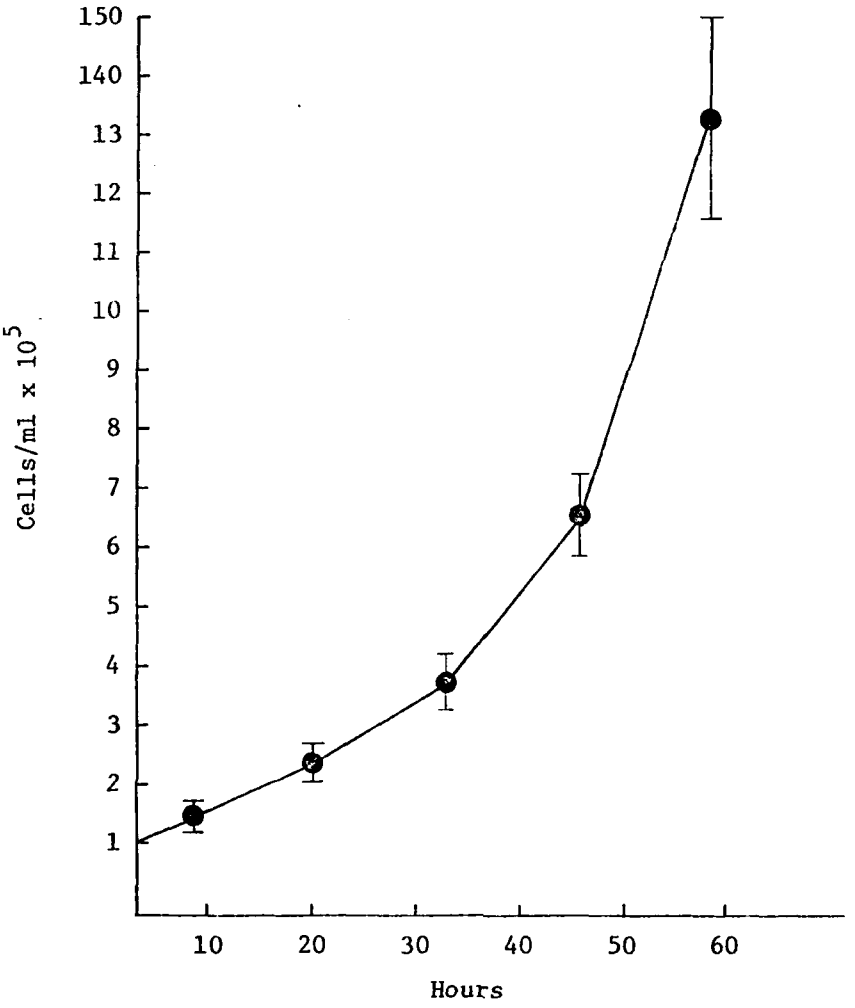
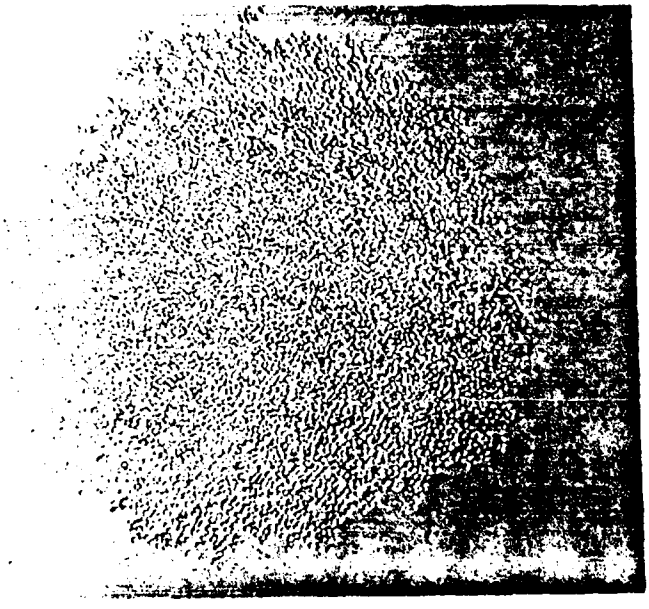


FIGURE 13. GROWTH OF AN SP2/0 CULTURE BEGUN AT A NOMINAL DILUTION OF 1 x 10^5 CELLS/ML. DATA ARE MEANS ± STANDARD ERRORS FOR EIGHT HEMACYTOMETER COUNTS.



Literature Cited

Andreadis, T.G. 1968. Life Cycle and Epidemiology of *Amblyospora* sp. (Microspora: Thelohaniidae) in the Mosquito *Culex salinarius* Coquillett. Ph.D. Diss., Univ. of Fla., Gainesville, Fla. 59 pp.

Andreadis, T.G. and D.W. Hall. 1979a. Development, ultrastructure, and mode of transmission of *Amblyospora* sp. (Microspora) in the mosquito. J. Protozool. 26, 444-452.

Andreadis, T.G. and D.W. Hall. 1979b. Significance of transovarial infections of *Amblyospora* sp. (Microspora: Thelohaniidae) in relation to parasite maintenance in the mosquito *Culex salinarius*. J. Invertebr. Pathol. 34, 152-157.

Borovsky, D. 1981. In vivo stimulation of vittellogenesis in *Aedes aegypti* with juvenile hormone, juvenile hormone analogue (ZR515) and 20-hydroxyecdysone. J. Insect Physiol. 27, 371-378.

Hagedorn, H.H., J.G. Kunkel, and G. Wheelock. 1978. The specificity of an antiserum against mosquito vitellogenin and its use in a radio-immunological precipitin assay for protein synthesis. J. Insect Physiol. 24, 481-489.

Hagedorn, H.H., J.D. O'Conner, M.S. Fuchs, G. Sage, D.A. Schlaeger, and M. K. Bohm. 1975. The ovary as a source of ∝-ecdysone in an adult mosquito. Proc. Nat. Acad. Sci., U.S.A. 72, 3255-3259.

Hazard, E.I., T.G. Andreadis, D.J. Joslyn, and E.A. Ellis. 1979. Meiosis and its implications in the life cycles of *Amblyospora* and *Parathelohania* (Microspora). J. Parasitol. 65, 117-122.

Hazard, E.I. and J. Weiser. 1968. Spores of *Thelohania* in adult female *Anopheles*: Development and transovarial transmission, and redescriptions of *T. legeri* Hesse and *T. obesa* Kudo. J. Protozool. 15, 817-823.

Kennett, R.H. 1980a. Freezing of hybridoma cells. In R.H. Kennett, T.J. McKearn, and K.B. Bechtol, Eds. Monoclonal antibodies. Plenum Press, New York. 424 pp.

Kennett, R.H. 1980b. Fusion by centrifugation of cells suspended in polyethylene glycol. In R.H. Kennett, T.J. McKearn, and K.B. Bechtol, Eds. Monoclonal antibodies. Plenum Press, New York. 423 pp.

Lea, A.O. 1963. Some relationships between environment, corpora allata, and egg maturation in aedine mosquitoes. J. Insect Physiol. 9, 793-809.

Lea, A.O. 1969. Egg maturation in mosquitoes not regulated by the corpora allata. J. Insect Physiol. 15, 537-541.

Lea, A.O. 1972. Regulation of egg maturation in the mosquito by the neurosecretory system: the role of the corpus cardiacum. Gen. Comp. Endocr. Suppl. 3, 602-608.

Littlefield, J.W. 1964. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145, 709-710.

McKearn, T.J. 1980. Cloning of hybridoma cells by limiting dilution in fluid phase. In R.H. Kennett, T.J. McKearn, and K.B. Bechtol, Eds. Monoclonal antibodies. Plenum Press. New York. 424 pp.

Ohtaki, T. and C.M. Williams. 1970. Inactivation of α-ecdysone and cyasterone by larvae of the flesh fly, *Sarcophaga peregrina* and pupae of the silkworm, *Samia cynthia*. Biol. Bull. Wood's Hole 138, 326-333.

Sprague, V. 1977. Classification and phylogeny of the microsporidia. In Comparative Pathobiology, L.A. Bulla and T.C. Cheng, Eds. Vol. 2. Systematics of the Microsporidia. Plenum Press. New York. pp. 1-30.

Weiser, J. 1977. Contribution to the classification of microsporidia. Vest. Csl. Spol. Zool. 10, 245-272.

OFFICE OF NAVAL RESEARCH
NAVAL BIOLOGY PROJECT
STANDARD DISTRIBUTION LIST

Number of Copies

(12) Administrator
Defense Technical Information Center
Cameron Station
Alexandria, VA 22314

(6) Director
Naval Research Laboratory
Attn: Technical Information Division
Code 2627
Washington, DC 20375

(3) Office of Naval Research
Naval Biology Project
Code 443
800 N. Quincy Street
Arlington, VA 22217

One copy to each of the following:

Office of Naval Research
Code 200
800 N. Quincy Street
Arlington, VA 22217

Office of Naval Research Eastern/
Central Regional Office
Building 114, Section D
666 Summer Street
Boston, MA 02210

Office of Naval Research Branch
Office
536 South Clark Street
Chicago, IL 60605

Office of Naval Research Western
Regional Office
1030 East Green Street
Pasadena, CA 91106

Technical Library
U.S. Army Natick Laboratories
Natick, MA 01760

Dr. A. L. Slafkosky
Scientific Advisor, Commandant of
Marine Corp (Code RD-1)
Washington, DC 20380

Assistant Commander for Research &
Development
Code 03
Naval Facilities Engineering Command
200 Stovall Street
Alexandria, VA 22332

Biological Sciences Staff
Code 112B
Naval Facilities Engineering Command
200 Stovall Street
Alexandria, VA 22332

Scientific Library
Naval Biosciences Laboratory
Naval Supply Center
Oakland, CA 94625

STANDARD DISTRIBUTION LIST (Cont'd)

Commander
Army Research Office
Research Triangle Park, NC 27709

National Environmental Research Center
Edison Water Quality Research Division
Edison, NJ 08817

Technical Advisory Division
National Marine Fisheries Service
Department of Commerce
Washington, DC 20235

Head, Disease Vector Control Section
BUMED (MED-514)2)
Department of the Navy
Washington, DC 20372

Matthew Stevenson
National Academy of Sciences
Room JH 538
2101 Constitution Avenue
Washington, DC 20418

Commanding Officer
Navy Environmental Health Center
Naval Station
Norfolk, VA 23511

Director
National Library of Medicine
8600 Wisconsin Avenue
Bethesda, MD 20014

Chief, Entomology Research Branch
Prevention Medicine Division
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, MD 21701

David W. Taylor Naval Ship Research & Development Center
Code 2831
Annapolis, MD 21402

Health Effects Research
Environmental Protection Agency
ORD 683401
401 M Street, SW
Washington, DC 20468

Commanding Officer
Naval Medical Research & Development Command, Code 46
National Naval Medical Center
Bethesda, MD 20014

Commandant, DWT
U.S. Coast Guard
400 Seventh Street, SW
Washington, DC 20511

Commandant, DAS
U.S. Coast Guard Research & Development Center
Avery Point
Groton, CT 06340

Commander Naval Oceanography Command
NSTL Station
Bay St. Louis, MS 39529

Officer in Charge
Navy Disease Vector Ecology & Control Center
Naval Air Station
Alameda, CA 94501

Officer in Charge
Navy Disease Vector Ecology & Control Center
Naval Air Station
Jacksonville, FL 32212

David W. Taylor Naval Ship Research & Development Center
Code 286
Annapolis, MD 21402

David W. Taylor Naval Ship Research & Development Center
Code 2856
Annapolis, MD 21402

STANDARD DISTRIBUTION LIST (Cont'd)

Alexander, Dr. Martin
Department of Agronomy
Cornell University
Ithaca, New York 14850
NR 205-052
N00014-78-C-0044

Boush, Dr. G. Mallory
University of Wisconsin
College of Agriculture and Life Sciences
Department of Entomology
Madison, Wisconsin 53706
NR 159-002
N00014-80-C-0080

Colwell, Dr. Rita R.
Department of Microbiology
University of Maryland
College Park, Maryland 20742
NR 153-081
N00014-75-C-0340

Crossley, Dr. D. A.
University of Georgia
Department of Entomology
Athens, Georgia 30602
NR 205-058

DePalma, Mr. John
National Space Technology Lab
Building 1105, Room C-316
NSTL Station, Mississippi 39522
NR 205-006
N0001479WR90115

Fahlstrom, Mr. G. B.
Osmose Wood Preserving Company
980 Ellicott Street
Buffalo, New York 14209
NR 205-036
N00014-80-G-0033

Findl, Mr. Eugene
BioResearch, Incorporated
315 Smith Street
Farmingdale, New York 11735
NR 135-901
N00014-78-C-0715

Hall, Dr. Donald W.
Associate Professor
Department of Entomology and Nematology
University of Florida
Gainesville, Florida 32611
NR 205-055
N00014-80-C-0172

*Khalil, Dr. G.
Naval Medical Research Unit Number 3
Medical Zoology Department
FPO New York 09527
NR 205-040

Linley, Dr. John R.
Florida Medical Entomology Laboratory
P.O. Box 520
Vero Beach, Florida 32960
NR 205-050
N00014-80-C-0261

Mitchell, Dr. Ralph
Harvard University
Division of Engineering and Applied Physics
Cambridge, Massachusetts 02138
NR 205-002
N00014-76-C-0262

Nash, Dr. Claude H. III
SK&F Laboratories
P.O. Box 7929
Philadelphia, Pennslyvania
NR 205-005
N00014-79-M-0057

* Address outer envelope as follows: Commanding Officer, NAMRU-3, FPO, NY 09527. Address inner envelope as shown in above listing.

Belmore, C. Irene
Battelle Columbus Laboratories
William H. Clapp Laboratories, Inc.
Washington Street
Duxbury, Massachusetts 02332
NR 205-034
N00014-79-C-0067

Romanovsky, Dr. V.
Centre de Recherches et d'Etudes
Oceanographiques
73-77, rue de Sevres
92100 Boulogne, France
NR 205-016
N00014-78-M-0034

Ross, Dr. Mary H.
Department of Entomology
College of Agriculture and Life
Sciences
Virginia Polytechnic Institute and
State University
Blacksburg, Virginia 24061
NR 205-028
N0C014-77-C-0246

Sonenshine, Dr. D. E.
Old Dominion University
Biology Department
Norfolk, Virginia 23508
NR 205-059

Vedros, Dr. Neylan A.
Scientific Director
Naval Biosciences Laboratory
Naval Supply Center
Oakland, California 94625
NR 205-001
N00014-75-C-0774

Weidhaas, Dr. D. E.
Insects Affecting Man and Animals
Research Laboratory
USDA-SEA-AR, Southern Region
1600 S.W. 23rd Drive
P.O. Box 14565
Gainesville, Florida 32604
NR 139-005
N00014-79-F-0070


DAT
FILM