1971


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FAUST III, Charles Carroll, 1925-
STUDIES ON THE CYTOPLASMIC INCOMPATIBILITY SYSTEM
IN CULEX PIPIENS: A GENETIC AND
ELECTROPHORETIC STUDY OF MITOCHONDRIAL
STRUCTURAL PROTEINS.

The Louisiana State University and Agricultural
and Mechanical College, Ph.D., 1971
Zoology

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STUDIES ON THE CYTOPLASMIC INCOMPATIBILITY SYSTEM IN
CULEX PIPIENS: A GENETIC AND ELECTROPHORETIC STUDY
OF MITOCHONDRIAL STRUCTURAL PROTEINS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Zoology and Physiology

by

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December, 1971
ACKNOWLEDGMENT

Appreciation is acknowledged to Dr. Wilbur French for his direction of this study; to the members of my committee for their constructive criticism; to the Department of Microbiology and the Department of Plant Pathology for use of their ultracentrifuges; and to my wife and children for their patience, understanding and support throughout this study.
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ABSTRACT

Four strains of Culex pipiens complex were selected for study of cytoplasmically inherited differences. These differences are phenotypically expressed as the ability or inability of a particular strain to produce fully fertile and viable offspring in another strain. Three theoretical crossing possibilities exist: (1) complete compatibility in both directions, (2) complete compatibility in one direction with sterility in the other, and (3) complete incompatibility in both directions. Compatible crosses produce fully fertile and viable offspring while incompatible crosses produce full lethality. However, many populations when studied extensively show a partial incompatibility.

Appropriate crosses, reciprocal crosses, test crosses and backcrosses were made to establish the degree of compatibility or incompatibility expressed by the four strains.

There are at least three different cytoplasmic factors present in the involved organisms. Mitochondria were extracted and fractionated for the structural proteins. This fraction, when purified and treated with performic acid, was analyzed by polyacrylamide gel electrophoresis. There were no observable differences in the structural proteins of the four strains.
INTRODUCTION

Cytoplasmic Inheritance

"The first example of a nonchromosomal gene was described in 1908 by the German botanist Carl Correns, one of the three biologists who had rediscovered the law of inheritance enunciated in 1865 by Gregor Mendel. Correns thought it likely that all organisms had more than one genetic system, and he and his students investigated the problem in a number of plant species (81)." However, the bulk of genetic investigation was destined to be in the direction of chromosomal or nuclear genetics, for this system is more orderly, and at the same time, more accessible than the nonchromosomal system (81, 95).

During the first half of this century, a number of traits was described which did not follow normally accepted Mendelian inheritance patterns. These traits were expressed by diverse groups of organisms such as flowering plants, protista and insects (81). Concurrent with the investigations of cytoplasmic inheritance was the study of the mitochondria. Descriptions of these structures began about 1850, but actual knowledge progressed slowly until near the turn of the century, at which time improved staining techniques permitted significant advances to be made. Up until the time of World War I, many early cytologists considered the role of mitochondria to be genetic (49). Following World War II, the full realization that these structures were directly involved in aerobic respiration gave direction and stimulation to research involving these
The mitochondrion has, as it is understood today, several functions in the eucaryote cell. One of these is the genetic role in mitochondrial continuity (95).

**Recent Reports of Cytoplasmic Inheritance in Metazoan Systems**

Several cytoplasmically inherited or extrachromosomal traits have been reported in *Drosophila*; CO₂ sensitivity (47), "sex ratio" (12), delta element (62, 63), and nonchromosomal freckled (5). The CO₂ sensitivity is now considered to be due to an infectious microorganism (48). The sex ratio condition can be transmitted both interspecifically and intraspecifically by injection (56) of ooplasm from strains which exhibit this condition. Spirochaetes have been shown to be the infectious cytoplasmic agents involved in this condition (73). Of particular interest is the delta element (62, 63) whose phenotypic expression is a segregation distortion. The interpretation of this distortion is that certain second chromosomes are sensitive to the killing action of delta. An organism homozygous for the sensitivity would be more susceptible than a heterozygote of sensitive and insensitive chromosomes. The trait is transmitted through both male and female members of the affected strain. Transmission by males, however, is considered to be below the threshold of killing action, but could not be eliminated from the sensitive line by repeated backcrosses of sensitive males and insensitive females. The exact nature of this agent is not fully understood (64). Freckled, in the absence of the second chromosomal gene for freckled, appears to be cytoplasmically transmitted (5).
Ryan and Saul (80) describe a post-fertilization effect in Mormoniella, which follows a maternal line of inheritance. Mormoniella, as most Hymenoptera, produce diploid females and haploid males as a consequence of fertilized or unfertilized eggs. Fertilized females produce about 85% females and 15% males, while eggs of virgin females give rise to 100% males. Several stocks of this tiny wasp have been found to produce abnormal ratios of offspring (exclusively or predominantly male) when the females of these stocks are bred to the wild type. The suggested mode of transmission is cytoplasmic because of the maternal inheritance, but the nature of the causative agent is not indicated.

Cytoplasmic determiners are being proposed as the mode of inheritance of several factors currently reported in mammals. One of these factors, low fertility, was recently described as existing in a strain of laboratory mice at the Nagoya University in Japan (96). Females of the strain produced a mean litter size of 1.1 when bred with males of a second strain; 2.6 when bred to a third strain; the reciprocal cross-yield was 7.2 and 7.7, respectively. The depressed fertility resulted from embryonic death three to four days following copulation. No observable malformation appeared in the few embryos which reached term, nor were there any malformed or atrophied foeti in the uteri of these females. Death apparently occurred prior to or during the process of implantation.

Several possibilities of cytoplasmic inheritance in man have been proposed: an inherited form of melanoma (2), anencephaly and spinabifida (67). The proposals are based upon a higher incidence of the condition
being transmitted by carrier or afflicted mothers than that transmitted by carrier or afflicted fathers. Possibilities of a cytoplasmic inheritance in man are strengthened when consideration is given to the studies in other advanced eucaryotic organisms (29). These studies have indicated that the phenotypic manifestation of mitochondrial complementation is gross teratological growth. This aberrant growth then would be the result of different cells within an individual containing various proportions of alternative forms of complementing mitochondria.

**Cytoplasmic Inheritance in Mosquitoes**

The existence of a nonreciprocal crossing ability in the breeding of mosquito species and subspecies has been shown in *Aedes* (10, 19, 87, 93), *Anopheles* (79), and the *Culex pipiens* complex (6, 17, 18, 22, 23, 24, 26, 27, 28, 30, 31, 39, 40, 41, 42, 43, 45, 46).

Several authors (31, 86, 93) considered the possibility of a cytoplasmic factor as the agent of interspecific sterility, but it was not until several years later that Laven (40) published his now classic paper. According to him, there are three possible crossing relations:

(1) successful crossing in both directions

(2) successful crossing in one direction, but crossing sterility in the reciprocal direction

(3) sterility in both directions.

The original work included mosquitoes from seventeen different geographical regions, and the animals were found to be grouped into nine crossing types. The nine groups offer seventy-two theoretical combinations, and of these possibilities only forty-four were completed
in both directions. Another twelve combinations were tested in one direction.

Twenty different crossing types (42, 43) are now known in the *Culex pipiens* complex. These types are predicated upon the ability of males of a particular group to produce fertile and viable hybrid offspring in crosses with females of other groups, or to produce fertile and viable offspring when inseminated by males of other groups (40). It is not known whether the crossing types are the result of a single plasmagene with numerous mutations, or of several independent plasmagenes.

It has been reported in the literature (43) that there is no change of the one reported criterion of cytoplasmic inheritance determinants, and consequently, of the crossing type to a stepwise change. Factors with an intermediate effect have never been observed according to Laven. A 'mutation' of a given cytoplasmic type to a different type leads to a clear-cut result in a single step. This conclusion is *non sequitur* on three counts.

1. Factors with intermediate effects have been reported (6, 17, 18, 23).

2. Current concepts concerning the relationship of mitochondria to cytoplasmic inheritance, to be discussed later, disposes of this proposed criterion.

3. Segregation of cytoplasmic genes have been reported (23).

French (24), using precise genetic manipulations involving the red eye marker gene, has changed many of the early concepts concerning cytoplasmic incompatibility in the *Culex pipiens* complex. He was able to show that intermediate effects are in evidence and that the inheritance of these effects is through the cytoplasm.
Males from a single female should contain a random sample of the cytoplasmic genes from their mother's germinal cytoplasm, and individual sperm will represent limited subsamples of the cytoplasmic genes of the male's germinal cytoplasm. Additionally, daughters of a single female should also contain random samples of the maternal germinal cytoplasmic genes. The cytoplasmic genome of a second generation female can be determined by test crossing her male offspring. Selection for cytoplasmic genes then becomes a matter of utilizing progeny from those females which show an increase or decrease in the degree of incompatibility when compared to the degree of incompatibility of their mother. The females which show the appropriate increase or decrease are utilized for further generations of selection.

Two Aswan lines were realized using this technique. One was completely incompatible when males of this line were crossed with females of the Theis strain, and the other line showed a high degree of compatibility in the test crosses. Crosses involving these two lines produced fully fertile and viable offspring in both crossing directions. However, in all the appropriate F1, F2 and backcrosses, the males when crossed with females of the Theis strain showed either a complete incompatibility or a high degree of compatibility when tested with the Theis females. The compatibility or incompatibility in these crosses was determined by the crossing types of the immediate maternal cytoplasm.

The results of this experiment are not in accord with the hypothesis that translocations, polarized segregation or infectious microbial agents are responsible for cytoplasmic incompatibility in the Culex pipiens complex.
In vitro complementation has been suggested as a method of studying mitochondrial polymorphism (60, 82, 83). Complementation analysis of mitochondria extracted from mosquitoes of different crossing types shows that differences exist in these mitochondria (24). Mixed mitochondria in various proportions produced patterns of enzyme activity which are significant and reproducible. Mitochondria from the mating types are different and when placed in close proximity to each other interact and alter their metabolic efficiency (29). This then suggests that the metabolic activity of mitochondria or mitochondrial derivatives present in the sperm would be altered when interacting with certain maternal cytoplasmics. Altered metabolic activity then is the possible mechanism of cytoplasmic male sterility in the Culex pipiens complex.

Classification of Mosquitoes

Speciation of the Culicidae is not completely understood even though they are probably the best known and most often studied of the order Diptera (37). The taxonomic classification recommended by Mattingly (58) will be utilized. His recommendation is that members of the Culex pipiens complex should be treated as a single polytypic species.

Biogenesis of Mitochondria

Theories concerning the origin of mitochondria are numerous and all have some supportive evidence (49). However, the origin of mitochondria from pre-existing mitochondria appears to receive more support today than other theories concerning the origin of these structures (34, 49, 95). There have been two outstanding studies supporting the theory
of division. The first was an electron microscopy study of a small single-celled marine alga, *Micromonas*, (57) which possess one nucleus, one chloroplast, and one mitochondrion; all three appear to divide synchronously at cell division.

The second study is convincingly in favor of mitochondrial division. Luck (55) grew a choline-requiring strain of *Neurospora crassa* on a medium which contained radioactive choline. Radioactive choline was incorporated into the mitochondrial membrane. After incorporation, the cultures were transferred to a non-radioactive medium and permitted to grow. When the culture had doubled in size, the radioactivity per mitochondrion as determined by autoradiographic means had decreased to one-half. Additional observation on three mass doubling cycles showed a random distribution of the radioactive label within the mitochondria.

**Origin of Mitochondrial Protein**

Mitochondrial protein is evidently derived from two sources, the nucleus and the mitochondria (32). Demonstration of *in vitro* and *in vivo* incorporation of labeled amino acids by isolated mitochondria indicates that the insoluble protein fraction of the mitochondria shows the greatest activity (8, 36, 76, 77, 94) of all the protein fractions of the mitochondria. The purified structural proteins from these organelles account for approximately 75% of the total radioactivity incorporated in these organelles (36, 77). These data suggest that structural protein is coded for and synthesized within the mitochondria (36) while other fractions such as cytochrome a (8, 36), cytochrome c (8, 36, 78), and malic dehydrogenase (78) are synthesized in the extra mitochondrial cytoplasm, but are ultimately incorporated into the mitochondria.
Results utilizing chloramphenicol, which inhibits isolated mitochondrial protein synthesis (3, 38), and cycloheximide, an inhibitor of protein synthesis within the cytoribosome-cell sap system (3, 53), suggests that cytochromes a and b are synthesized within mitochondria. However, Ashwell and Work (4) caution that "one of the main difficulties in analyzing the effects of choramphenicol and cycloheximide in whole cells arises from the probability that neither protein-synthesizing system operates independently of the other."

In vivo studies by Beattie, et al. (8) have shown that rat liver and kidney mitochondria, labeled with radioactive leucine or valine, exhibit variations in the time of incorporation of labeled amino acids within the various mitochondrial fractions. The incorporation of radioactive amino acids into the liver mitochondrial structural proteins, when compared to whole mitochondrial incorporation, showed a difference which was considered insignificant by the authors. This insignificant difference was found for all time periods from two minutes to eight hours of incorporation, and indicates that most of the activity is incorporated into the structural proteins.

An analysis of these facts supports the hypothesis that while cytochromes a and b (3, 8, 53) and contractial proteins (8) may be coded for by mitochondrial DNA, structural protein is generally accepted as being a product of mitochondrial protein synthesis. Isolated mitochondria incorporate a preponderance of labeled amino acid in this structural protein fraction (36, 77). This structural protein fraction plays a critical role in the organization and assembly of the enzymes within the mitochondria (98).
Gel Electrophoresis

The technique of gel electrophoresis progressed rapidly following the development of zone electrophoresis on starch gel by Smithies (85), and the subsequent development of polyacrylamide gel for zone electrophoresis by Davis and Ornstein (16). Polyacrylamide gel electrophoresis is a very effective technique for resolving high molecular weight proteins. This resolution results from different electrical charges on the proteins and the sieving action of the gels (16, 75).

Polyacrylamide gel electrophoresis has been utilized to separate the proteins from the four complexes of the mitochondrial electron transfer chain (92). Additional use of polyacrylamide gel has been made in the fractionation of the proteins of the inner and outer membrane proteins of rat liver mitochondria (84), and in the separation of an apparent homogenous structural protein from beef heart mitochondria into four fractions (34, 51).

Rationale

Laven's genome replacement (40), and cytoplasmic analysis within the Aswan strain by French (23), disposes of nuclear control as the causative agent in Culex cytoplasmic incompatibility. In addition, this evidence eliminates the possibility of translocations, polarized segregation, and symbiotic organisms as agents of this incompatibility. Further evidence has shown that "cytoplasmic genes are expressed, at the molecular level, as polymorphic differences in mitochondria (28)." Genetic crosses in this investigation support the hypothesis that cytoplasmic inheritance
in the *Culex pipiens* complex is associated with polymorphic forms of mitochondria.

Demonstration of mitochondrial DNA by electron microscopy and by autoradiography of tritiated thymidine labeled cells has been shown. Indications are that the presence of mitochondrial DNA is extensive throughout the Animal Kingdom (35, 66, 68, 69). The existence of polymorphic forms of the mitochondria which can be associated with clear-cut phenotypic differences provides an exceptional and unique model system for molecular investigation of the non-nuclear inheritance system in advanced eucaryotic animals (29, 30).

It has been shown that mitochondrial structural protein is coded for and synthesized within the mitochondria (36), while other mitochondrial protein fractions may be synthesized outside and then incorporated into the mitochondrial membranes (8, 36, 78). Mitochondrial structural proteins have a critical role in mitochondrial enzyme assembly and organization and show variations in the amino acid composition in different cytoplasmic strains of *Neurospora* (98). A molecular investigation of the mitochondrial structural protein of cytoplasmically different strains of the *Culex pipiens* is, therefore, logical, especially when multiple components of beef heart mitochondrial structural protein has been demonstrated using polyacrylamide gel (51).
MATERIALS AND METHODS

Stocks

The organisms involved in this study are of the *Culex pipiens* complex, one of the eukaryotic animal species with a known cytoplasmic inheritance system. Using the two letter designation system of Laven, the four strains utilized were as follows:

(1) Ha - Hamburg, Germany. This white-eyed autogenous strain came to our laboratory from A. Ralph Barr, University of California, Los Angeles, California, who obtained his stock from Hannes Laven, Max-Planck-Institut fur Biologie, Tubingen, Germany. Laven received his animals from F. Weyer, Tropeninstitut, Hamburg, Germany where they had been bred continuously since the 1930's.

(2) Pa - Paris, France. Another autogenous strain with a red-eye marker gene was obtained through the auspices of Barr, but originally obtained by Laven from the Institute Pasteur, Paris, France.

(3) HC - Baton Rouge, Louisiana. A strain taken near the Baton Rouge Campus of Louisiana State University and selected for incompatibility. The original animals taken by W. L. French several years ago showed a partial incompatibility, and since that time have been maintained through close inbreeding, with selection toward an increased incompatibility. This strain has the red-eye marker gene and is autogenous.
Bl - Baton Rouge, Louisiana. A second strain taken in the vicinity of Baton Rouge and selected for a high percentage of hatch.

Rearing of Stocks

All animals were mass reared in the laboratory using the following methods:

1. **Cages**: After emergence, adults were placed in cages prepared from round half gallon ice cream cartons, with net sleeves enclosing the open end. The sleeves permitted easy access for daily removal of a water-filled finger bowl, the depository for egg masses. Additionally, honey-soaked cotton was placed in each cage.

2. **Blood Feeding**: The blood meal was withheld for a period of three to five days following emergence to allow insemination. This time lapse permitted a majority of the females to become inseminated before consumption of blood.

Doves, pigeons, and quail served as the blood meal source for *Culex pipiens* has a preference for avian blood. The birds were restrained by being placed in a small box and having the wings and feet tied, or by use of a special restraining board designed by W. L. French. Where feasible, the restraining board was used, for it provided a relative degree of comfort for the bird, yet did not permit excessive movement.

A few feathers were removed from the breast of birds restrained in boxes, or from the backs of those placed on the special boards. The boxes were placed within the cages, while the boards were placed on top, so the mosquitoes could feed through the netting.
This last method proved superior in that entrance into the cage was eliminated, thereby decreasing the opportunity for contamination and/or loss of adults.

Blood feeding was accomplished during the daylight hours by placing the cage with the affixed bird in a cabinet for six to eight hours of darkness. While it is not absolutely necessary, darkness appears to provide a higher percentage of blood fed females.

The birds were held in a special room with twenty-four hours of daylight in order to increase the likelihood of quietness, or even sleep, during the dark blood feeding period. This lack of bird movement provided the mosquitoes a better opportunity to achieve a full blood meal.

(3) Eggs. Three to four days after the intake of a blood meal, females began to deposit egg rafts in the water-filled finger bowls.

Bowls were changed each day and the individual egg masses placed in shell vials, where hatching usually began the day after isolation. The isolated egg rafts, following a sufficient time period for hatch, were counted and scored for hatch, unembryonated, embryonated but unhatched, and embryonated with distorted eyes.

(4) Larvae. As each egg mass was segregated, a small amount of food was sprinkled on the water for the future hatching larvae, and the shell vial was plugged with cotton. All four larval stages were fed an equal mixture, by weight, of Kellogs' Concentrate and Kretschmer's Wheat Germ.
After hatching, the larvae from a single egg mass were placed in a quart milk bottle at the rate of approximately fifty larva per bottle. Each day the water level was raised about one inch, the water swirled, and a pinch of food added. One grain of puffed wheat was also contributed to each bottle, so that a constant supply of food was available on the top of the water.

(5) Pupae. Pupation normally occurred ten to twelve days from the time the eggs began to hatch. Pupae from an egg raft with a high percentage of hatch were periodically isolated for the purpose of test crosses.

(6) Adults. Adults were removed each day and placed in appropriate cages to await blood feeding or grinding for electrophoresis. Only those egg rafts which exhibited a very high degree of hatch and the proper compatibility or incompatibility with respect to a reference strain were utilized in the maintenance of stocks.

**Line Development**

Mosquitoes have been continuously tested over the past two years for the degree of fertility within each strain and the degree of compatibility or incompatibility expressed in relation to other lines. Some 1800 egg rafts have been counted in the course of this study. Another two to three thousand egg rafts have also been scanned.

Males of a given female should possess a random sample of their mothers' germinal cytoplasmic genes, and the sperm from these males should represent a limited subsample of that male's germinal cytoplasmic genes. Daughters from this same female will also contain random samples of their mothers' germinal cytoplasm (23).
Utilizing this information, three of the four strains in this investigation were developed. Egg masses from the Pa, HC, and Ha lines were checked each day. Those hatchlings which came from an egg mass with a high percentage of hatch were reared to adulthood and those which came from an egg mass with a low percentage of hatch were discarded. Siblings from the same egg mass were kept together and the container in which they were reared was appropriately marked. Through this method of breeding and selection, three of the four lines used in this investigation were developed. Periodically as the adults began to emerge, males are usually first, a few males of the Pa and HC lines were tested to the Ha line. Periodic tests were also made between the Pa and HC lines.

The Ha line was continuously selected for a high percentage of hatch and at the conclusion of the investigation showed an average 75.2% intrastrain hatch rate. In this investigation, as well as in other crosses involving this line, the cytoplasmic genes of the Ha line do not appear to be stable. The instability referred to here can only be stated for our population.

The Bl line was only recently introduced into the laboratory and tests show that it has a high degree of unilateral incompatibility to the other strains.

**Partial Incompatibility**

When interstrain crosses are made, compatible crosses produce fully fertile and viable offspring and incompatible crosses produce full lethality. If the degree of incompatibility is complete, then its
detection is quite simple, but if the degree of incompatibility is low, then more precise procedures are necessary for its detection (23).

In completely incompatible crosses, the sperm will initiate development of some eggs, but karyogomy will be blocked. These eggs will develop parthenogenetically. Most of these eggs will develop as haploids but the resulting larvae do not hatch. Exceptional cases of diploid parthenogenetic larvae apparently result from the union of the egg pronucleus and one of the polar bodies. These exceptional larvae do sometimes hatch. Use of the red-eye marker gene is useful in the detection of both haploid and diploid parthenogenetically developing larvae. When this marker gene is present only in the female member of a cross, parthenogenetically developing larvae will show red eyes as a result of their maternally inherited genome (23).

In partially incompatible crosses, some eggs will show that karyogomy has been completed. These larvae will be diploid and may or may not hatch. Unhatched larvae resulting from completed karyogomy will show the wild type eye color of their paternal parent through the egg membrane. Two types of unhatched larvae are expected in partially incompatible crosses, parthenogenetic developing larvae with red eyes, and diploid larvae with wild type eyes as a result of fertilization (23). Comparable results are obtained when the sex linked recessive white eye is used.

Selection for Electrophoresis

Females, whose tested brothers all indicated the proper compatibility or incompatibility, were permitted to interbreed with other males of the
same egg raft. The progeny of these females were then collected in preparation for gel electrophoretic study.

Mitochondrial Protein Fractionation

The solutions used in the preparation of mitochondrial protein fractions for electrophoresis are:

**Solution A:** 0.25M sucrose with 0.01M Tris Hydroxyamino Acid at a pH of 7.4 and kept at a temperature of 4°C.

**Solution B:** 0.9% Potassium Chloride brought to a temperature of 30°C before use.

**Solution C:** 0.6M Potassium Chloride brought to a temperature of 30°C before use.

**Solution D:** 0.25M sucrose.

**Solution E:** 5.0% Trichloroacetic Acid.

**Solution F:** 8M Urea with pH lowered to 5.5 with HCl.

**Solution G:** 9 ml. 88% formic acid plus 1 ml. 30% Hydrogen peroxide. Prepared fresh before use.

Animals destined to be electrophoresed were placed in the cold for 10 to 15 minutes, weighed and ground, using a cold mortar and pestle. Mitochondria were extracted (33) at approximately 4°C, using the following procedure:

1. Grind in 9x volumes of Solution A.
2. Centrifuge at 600 times G for 10 minutes.
3. Wash the pellet with 9x volumes of Solution A.
4. Repeat Step 2.
5. Combine supernatants from Steps 2 and 4.
6. Repeat Steps 3 and 4.
7. Combine supernatant with that of Step 5.

8. Discard pellet.

9. Centrifuge combined supernatants at 8,700 times G for 10 minutes.

10. Discard supernatant.

11. Wash the pellet with 4x volumes of Solution A.

12. Centrifuge at 8,700 times G for 10 minutes.

13. Repeat Step 11.

14. Centrifuge at 18,800 times G for 10 minutes.

The method of Beattie, Basford, and Koritz (8) for the fractionation of mitochondrial proteins was used, but with slight modifications. The modified procedure is:

1. Washed mitochondria were treated with 4x of solution B and placed in a 30°C water bath for 10 minutes.

2. Centrifuge at 17,500 times G for 10 minutes.

3. Supernatant containing soluble proteins and cytochrome C was dialyzed against distilled water.

4. Pellet treated with 4x of Solution C and placed in a 30°C water bath for 10 minutes.

5. Centrifuge at 27,000 times G for 10 minutes.

6. Supernatant containing contractile proteins was dialyzed against distilled water.

7. Pellet homogenized in Solution D at the rate of 20 mg. of protein per ml. of solution.

8. The following chemicals were added to the homogenate:

   Sodium Cholate - 2X  
   Cholic Acid Sodium Salt - 1X  
   Sodium Duodecil Sulfate - 0.75X  
   Sodium Dithonite - A few grains  
   Ammonium Sulfate - Taken to 13%

9. Homogenate was then placed in the cold for 7 hours.
10. Homogenate was centrifuged at 34,800 times G for 10 minutes.

11. Pellet washed with 4x of Solution E.

12. Centrifuge at 34,800 times G for 10 minutes.

13. Repeat Steps 11 and 12.

14. Repeat Step 11 and incubate at 32°C for 15 minutes.

15. Repeat Step 12.

This crude mitochondrial "structural protein" fraction was then purified and treated using the procedure of Lenaz, Haard, Lauwers, Allmann and Green (51). The procedure follows:

1. Extracted "structural protein" was washed with neutral methanol.

2. Centrifuge at 34,800 times G for 10 minutes.

3. Resuspend pellet in distilled water.

4. Dialyze for 7 hours.

5. Freeze dry.

6. Suspend in Solution F, 10 ml. of protein per ml. of solution.

7. Stir for 30 minutes at room temperature.

8. Centrifuge at 100,000 times G for 15 minutes.

9. Repeat Steps 6, 7 and 8.

10. Repeat Steps 6, 7 and 8.

11. Suspend in distilled water and lyophilize.

12. Suspend in Solution G at the rate of 10 mg. per ml. of solution.

13. Keep at room temperature for 1 hour.

14. Reaction stopped by adding 20x volumes of distilled water.
15. Dialyze against distilled water.

16. Lyophilize.

**Electrophoresis**

Numerous attempts to use the EC Corporation vertical gel apparatus, varying buffers, gel pH, gel pore size, and migration direction, gave results which ranged from obscure to nil. These attempts led to the conclusion that the stringent solvents needed to dissolve the non-catalytic structural proteins of mitochondria would necessitate the use of disc electrophoresis with the gel polymerized in glass tubing, thus eliminating the danger of solvent reaction with the plastic walls of the vertical gel apparatus. The disc electrophoretic box was built according to a design suggested by W. L. French.

Electrophoresis was performed using the procedure of Takayama (92), as modified by Baum (7). Gel tubes were 5 mm. i.d. and 75 mm. l.; the gels consisted of 7.5% acrylamide, 35% acetic acid and 5M urea.

Gels were prepared using two stock solutions:

**Stock Solution A**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>6.0 gr.</td>
</tr>
<tr>
<td>N, N'-Methylene bis-acrylamide (Bis)</td>
<td>0.16 gr.</td>
</tr>
<tr>
<td>Urea</td>
<td>12.0 gr.</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>28.0 ml.</td>
</tr>
<tr>
<td>Water</td>
<td>to 60.0 ml.</td>
</tr>
</tbody>
</table>

**Stock Solution B**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulfate</td>
<td>0.40 gr.</td>
</tr>
<tr>
<td>Urea</td>
<td>12.0 gr.</td>
</tr>
<tr>
<td>Water</td>
<td>to 20.0 ml.</td>
</tr>
</tbody>
</table>
Gels were prepared by combining Stock A, Stock B and N, N, N\textsubscript{1}, N\textsubscript{1} - tetramethylethylene diamine (TMED) in the ratio of 12:4:0.1. Gel tubes were siliconized, stoppered and positioned in the upper chamber in preparation for receiving the gel solution. The stoppers, of a type described by Davis (15), have an inside diameter of about 6 mm. and were obtained from Moosa Memorial Hospital in Eunice, Louisiana. A 9" disposable pipette was used to fill the gel tubes. Each tube had 0.8 ml. of gel solution carefully placed in it by holding the tip of the pipette within the tube, and very close to the bottom, in order to minimize the occurrence of bubbles in the gel. A layer of 75% acetic acid was then sprayed over the top of the solution and the tubes placed in a 50°C water bath for 15 to 30 minutes for polymerization. The tubes were then removed, the wells rinsed with 75% acetic acid, and refilled with more of the same solution.

The protein sample was then carefully layered between the gel surface and the 75% acetic acid. The upper and lower buffer chambers were filled with 300 ml. and 800 ml. of 10% acetic acid, respectively; and the upper part of the apparatus, which houses both electrodes, was then placed in position within the lower tank. The lower reservoir electrode served as the cathode during the electrophoretic run. The current was maintained at 1.5 mA per tube until all of the tracking dye had migrated into the gel, at which time the current was increased to 5 mA per tube. Electrophoresis was continued until the dye had migrated approximately 25 mm. The current was then turned off, tubes were removed from the apparatus and placed in an ice bath for 15 to 20 minutes. Gels were removed from the tubes by ringing with a common needle probe and
stained in 0.25% amido black 10 B in 7.5% acetic acid which had been filtered before use. Gels remained in the stain solution approximately one hour, were washed with running water for one hour, and destained in 10% acetic acid using special destaining tubes.

The 7 ml. i.d. destaining tubes, their bottom plugged with 1 cm. of gel, were filled with 10% acetic acid and the stained gel lowered into the tube. The top of the tube was then fitted with a 6 mm. o.d. glass tube with rubber grommet. The telescoped destaining tube was then fitted into the electrophoretic apparatus, tanks refilled with 10% acetic acid, and the power adjusted to 10 ml. per tube. At the completion of destaining the power was turned off; gels were removed from the destaining tubes and stored in 10% acetic acid in special storage tubes.

**Purity of Mitochondrial Structural Protein**

One criterion of mitochondrial structural protein purity is its apparent homogeneous nature ascertained by polyacrylamide gel electrophoresis (51, 98). After electrophoresis, purified structural protein appears as a single protein band with an obvious lack of other bands, which would be indicative of contamination.
RESULTS

The basic crosses in Fig. 1 involve three strains of the *Culex pipiens* complex. Intrastrain hatch rates are shown in Table I, while crossing hatch rates are indicated in Table II. The Pa strain, with an intrastrain average hatch rate of 88.9%, and the HC strain, with an intrastrain 79.2% average hatch rate, show some degree of compatibility when the two strains are crossed. The two strains were closely inbred for two years. Pa females bred by HC males (Cross I) had an average of 71.6% of their eggs hatch, while the reciprocal cross, HC females times Pa males (Cross II), showed an average 88.3% hatch. The two strains are considered to be compatible.

The Ha strain shows an intrastrain average hatch rate of 75.2%. The white eyed Ha females times the red eyed Pa males (Cross III) are almost completely incompatible for only an average of 1.3% of the eggs from this cross hatched, while the white eyed Ha females times the red eyed HC males (Cross IV) gave a partially incompatible 32.0% average hatch. The use of the white eyed marker gene in the Hamburg strain shows in Table II that some gynogenetic development occurs, but a large number of distorted wild type eyes present in the unhatched larvae shows that both of these crosses are only partially incompatible. The results of the cross involving Ha females and Pa males show a higher degree of partial incompatibility than the results of the cross involving Ha females and HC males. In the first of these crosses only an average of 1.3% of the eggs hatched, while the second showed an average 32.0% hatch.
Fig. 1. Basic crosses involving the Pa, HC and Ha strains of the Culex pipiens complex. Solid or dashed arrows indicate the direction of the cross, i.e., from male members to female members involved. Crosses I and II are compatible, Crosses III and V are incompatible, and Crosses IV and VI are partially incompatible.
This then shows that the cytoplasmic determinants within the Pa and HC strains have a difference of at least two factors. This difference is expressed when males of these two strains are crossed out to the Ha strain. One of these factors is present in the Pa cytoplasm and is compatible when interacting with the HC cytoplasm, yet is incompatible when interacting with the Ha cytoplasm.

A combination of two determinants evidently exists in the HC cytoplasm. This combination is probably composed of the same factor found in the Pa cytoplasm, along with a different second factor. This is suggested by the results of the two outcrosses involving males of this strain. HC males bred to Pa females resulted in a 71.6% average egg hatch, while HC males times Ha females resulted in an average hatch rate of 32.0%. Approximately 70% of the HC sperm have a correct combination of factors which are compatible with Pa cytoplasm, while the remainder of these sperm have a combination which is incompatible. The outcross of this strain to Ha females shows the reverse of this condition. Approximately 30% of the HC sperm has a combination for compatibility in the Ha cytoplasm and the remaining sperm have a combination which is incompatible in the Ha cytoplasm. Results of the two outcrosses indicate that one of the factors of the combination is the same factor found in the Pa cytoplasm, while the other factor is of a different nature.

Ha females, when bred (Cross V) by F₁ males resulting from Cross I, produced essentially the same average hatch rate, 1.4%, as did Ha females in Cross III when bred by Pa males, 1.3%. The data from these two crosses (Table II) have a statistical t value of 0.27 with 44 degrees of freedom. The probability of a t value of this order is greater than 0.75.
Therefore, the two populations are statistically indistinguishable and there was no appreciable change in the Pa cytoplasm as a result of the Pa female times HC male cross.

The test cross of Ha females times HC males (Cross IV) shows an average 32.0% hatch rate and the F₁ test cross (Cross VI), which involved F₁ males resulting from Cross II, shows an average 27.5% hatch. The t value for these two crosses is 2.14 with 32 degrees of freedom. The probability of these data is a significant 0.04. We have, therefore, detected a possible change in the F₁ cytoplasm when compared with the original HC cytoplasm. The magnitude of this change is small when considering the 4,611 individual eggs involved in these two crosses. Several possibilities for this significant change are given in the discussion.

During the spring of 1970, after the Hamburg and Paris strains were received from California, crosses were made to determine the degree of compatibility existing between these two strains. That series of crosses, and subsequent series which were made during the period of investigation, shows that a change has occurred within the cytoplasmic inheritance system of these two strains. The average hatch rates for these crosses are shown in Table III. In the spring of 1970, crosses of Ha females and Pa males produced an average hatch rate of 32.0%. After close inbreeding and selection for high percentage of hatch within each strain, another series of crosses was made that same summer and the average hatch rate dropped to 20.4%. This is a highly significant (t = 4.89 with 22 degrees of freedom and P < 0.01) change in the two strains. Close inbreeding and selection was continued and a series of crosses made
during the fall of 1970 showed an average hatch rate of 5.7%. The statistical data for the change in average hatch rate from the summer of 1970 to the fall of 1970 are highly significant ($t = 14.0$ with 43 degrees of freedom and $P < 0.01$). Inbreeding and selection continued, and during the summer of 1971 a fourth series of crosses was made. The average hatch rate for this series was 1.3%. The change in the average hatch rate from the fall of 1970 to the summer of 1971 is also highly significant ($t = 8.8$ with 55 degrees of freedom and $P < 0.01$). These data show that a stepwise change has occurred in the cytoplasmic inheritance system of these two strains when Ha females were bred by Pa males.

An additional incompatibility factor is shown by the crosses in Fig. 2. Males of the Pa, Hc, and Ha strains are all incompatible when bred to females of the Bl strain. Average hatch rates for these crosses are shown in Table IV. Bl females times Ha males show an average 0.0% hatch rate, while HC males bred to Bl females show an average hatch rate of 0.1%. The Ha males and HC males in these two crosses could be considered as males from the same population for the statistical data of these crosses do not show a significant difference ($t = 1.26$ with 61 degrees of freedom and $P > 0.20$). Pa males times Bl females show a 0.3% average hatch rate. Statistical analysis of the crosses involving the HC males and Pa males does not show a significant difference ($t = 1.51$ with 67 degrees of freedom and $P > 0.17$). A significant difference is only evident when the two extremes are tested. The Bl females times Ha males and Bl females times Pa males show a statistically significant difference in the two crosses ($t = 2.29$ with 44 degrees of freedom and $P > 0.02$). This statistical difference is evidently small for only a
Fig. 2. Test crosses involving Ha, Pa and HC males with Bl females. All tests were incompatible.
30

0.3% average egg hatch separates the two crosses. There was a total of 6,232 eggs involved in these two crosses. The Ha, HC, and Pa strains show essentially the same degree of incompatibility in B1 cytoplasm.

A series of backcrosses was made to determine whether a maternal inheritance was involved in the incompatibility of the three strains in the B1 cytoplasm. B1 males were bred to females of each of the three strains; HC, Ha, and Pa. F₁ males from each of these crosses were then backcrossed to B1 females. Table IV shows that the results of these backcrosses have essentially the same incompatibility as the initial crosses. Crosses of B1 females times Ha males and B1 females times F₁ males of the Ha female times B1 male cross show an average hatch rate of 0.0%. B1 females times Pa males and B1 females times F₁ males of the Pa female times B1 male cross show a 0.3% difference in their average hatch rate. Crosses of B1 females times HC males and B1 females times F₁ males resulting from HC female times B1 male cross show an average 0.1% hatch rate.

Results of the above genetic crosses show that cytoplasmically inherited differences exist among the four strains of mosquitoes. There are at least three different factors which could be in varying proportions. The strains were subjected to polyacrylamide gel electrophoretic analysis.

Electrophoretic study of these cytoplasmic differences has progressed through a series of stages. The initial attempts involved one to ten female mosquitoes. These animals were ground in an appropriate solution and the cell debris removed by centrifugation. Samples of the supernatant were applied to microslide gels or to vertical gels and
subsequently electrophoresed. Results of these studies were varied and in many cases indistinct so that interpretation was impossible. It was not possible to increase the resolving power of this procedure.

It was decided that a difference which exists in the cytoplasm should be studied at the basic level, the mitochondrion. Mitochondria were extracted from young females which had been blood fed and held in cages for at least five days. They were thus permitted to digest the blood and to begin ovulation without the deposition of eggs. After this period of time, the abdomen of those blood fed females appeared swollen and yellowish white. Ovaries from these females were then removed and the mitochondria extracted from the ova. Although there was a visible quantity of mitochondria extracted from ten or more females, fractionation of this small mitochondria pellet never resulted in fractions which were resolvable by polyacrylamide gel electrophoresis.

Use of the present procedure, which involves one or more grams of adult mosquitoes, yields a much larger mitochondrial pellet. Protein fractions from this larger pellet usually range from one to two milligrams. The recommended ratio of protein sample to sample solvent is normally at least five mg. of protein sample per ml. of solvent (51, 92). A mitochondrial protein fraction of one milligram would thus be dissolved in 0.2 ml. of sample solvent. Protein fractions are recovered from the fractionation solution by dialysis and lyophilization. A one milligram mitochondrial protein fraction is thus dispersed over a relatively large area within the lyophilization tube, and a sample solution of less than 0.2 ml. is impractical.
The soluble fractions resulting from mitochondrial protein fractionation have shown that differences exist in the mitochondria of the four strains. Such a large wealth of proteins within the soluble fractions would require extensive fractionation before analysis. On the other hand, the techniques for the extraction and purification of the mitochondrial structural proteins are precise and well developed, and results obtained using these techniques are reproducible.

The purified structural proteins from the four strains of Culex studied show a similar single protein band (Plate 1) which migrated approximately 5 mm. from the origin under the experimental conditions employed. This is in accord with the results obtained from purified Neurospora mitochondrial structural protein. The amino acid composition of Neurospora purified mitochondrial structural protein of one mutant strain showed an apparent replacement of tryptophan by cysteine and the other mutant strain showed a lack of one tryptophan residue when these structural proteins were compared to the structural proteins of a normal strain. These Neurospora structural proteins were not oxidized by performic acid. Polyacrylamide gel electrophoresis of the Neurospora strains showed a single diffuse band (98). Purified heavy beef heart mitochondrial structural protein which had not been subjected to performic acid oxidation also showed a single band (51) on polyacrylamide gel. When this purified heavy beef heart mitochondrial structural protein was treated with performic acid the oxidized purified structural protein showed four bands on polyacrylamide gel.

Mosquito purified mitochondrial structural proteins which had been performic acid oxidized were resolved by polyacrylamide gel electrophoresis.
into three discernible protein bands (Plate 2). Although there are at least three, and possibly four, cytoplasmic types (see discussion) in the four strains of mosquitoes investigated, there was no observable difference between the strains when oxidized mitochondrial structural proteins of these four cytoplasmically different strains were analyzed by polyacrylamide gel electrophoresis. These results are similar to the results obtained from the electrophoretic studies of heavy beef heart mitochondria which showed four protein bands (51).

A period of almost two years was required for the development of the many necessary techniques of this analysis. After perfection of the complete analytic system, three generations of each of the four strains were subjected to that analysis and the results, which were reproducible, showed no detectable difference within the four strains under study.
DISCUSSION

French has shown that translocations, polarized segregation and microbial agents are not involved in the cytoplasmic inheritance of the Culex pipiens complex (23). Some additional support is given his findings when analysis is made of the crosses involved in this investigation.

Variations in the expected ratio of viable to nonviable gametes occur when translocations are the mechanism of sterility in insects. Curtis (13) discusses several possible means of shifting the expected ratio. These shifts would be in the nature of a repair mechanism and the expected 1 viable to 2 inviable gamete ratio would be changed to a 1 viable to 1 inviable ratio. However, Laven (44) found a system of translocations in Culex pipiens which shows a variation in the fertility of involved males. The translocations were closely linked with the gene for maleness and fertility of these males varied between 26 and 82 percent of the normal.

It is possible to visualize the action of a translocation in the HC strain which would produce an average 32.0% hatch when sperm from that strain were introduced into the Ha strain (Cross IV). The problem becomes complex when we consider that this same translocation or translocation series must increase its lethal capacity to an average 0.1% hatch in the Bl strain and then depress its lethality to permit a 71.6% average hatch when crossed to the Pa strain. Neither crosses of individuals nor series of crosses can be reconciled with this mode of action.
The same line of reasoning must then be applied to the Pa strain. Its system of translocations must be varied enough to permit an average 88.3% hatch production in the HC strain and yet be stringent in the Ha and Bi populations.

A translocation system would be rare which could meet the requirements needed to explain the results obtained in the crosses (Figs. 1 and 2) of this investigation.

Assuming a system similar to the extrachromosomal element delta in *Drosophila* (62), there is no evidence to support this type of nucleo-cytoplasmic interaction. Delta induces a type of segregation distortion in the second chromosomes of certain lines of *Drosophila*. The segregation is interpreted as a sensitivity of certain second chromosomes to the extra chromosomal element delta, so that homozygous insensitive or heterozygous insensitive and sensitive combinations of the second chromosome are not affected. Homozygous sensitive second chromosomes are lethal, for the delta element induces frequent lethal mutations at definite regions of sensitive chromosomes (63). The delta element is found in certain strains of *Drosophila* that carry homozygous insensitive or heterozygous insensitive and sensitive combinations of their second chromosomes. Out-crosses of these strains will show either a 50% reduction of offspring, as in an out-cross involving a homozygous carrier and a homozygous sensitive noncarrier, or a 75% reduction of offspring, as would be expected from an out-cross involving a heterozygous carrier and a homozygous sensitive noncarrier.

If the cytoplasmic incompatibility system in the *Culex pipiens* complex was to have a nucleo-cytoplasmic interaction like that involving
the delta element of *Drosophila*, the following conditions would be necessary.

The assumed element delta would necessarily be present in the Ha strain for this is the cytoplasm in which incompatibility results. If this strain were heterozygous for sensitive and insensitive chromosomes, we could well account for the average 75.2% intrastrain hatch rate. Heterozygosity must be ruled out, however, for out-crossing with the Pa strain produces only an average 1.3% hatch. Even if the Pa strain were to be homozygous sensitive, we could expect an approximate 25% hatch. A more strenuous condition would have to be in effect.

The delta element would have to be present in the Ha strain and its effective action would be deleterious to any developing embryo which was heterozygous for sensitive and insensitive chromosomes. It could only be carried then by an organism which was homozygous insensitive for the involved chromosome pair. The Ha strain would then be homozygous insensitive to the delta element and the Pa strain would be homozygous sensitive. In this cross the resulting zygotes would be heterozygous and, therefore, lethal. The HC strain must then possess a semi-heterozygous condition for the same pair. Approximately 30% of the members of the involved chromosome pair must be insensitive to the action of the hypothetical delta element. This would permit then the development of approximately 30% of the resulting larvae of Cross IV to hatch. The hypothesis begins to break down when we consider that the heterozygous F₁ organisms (Pa/HC) resulting from Cross I would be expected to carry an increased number of insensitive chromosomes and Cross V would show a substantial increase over the average 1.3% hatch of Cross III. This is not the case.
A third hypothesis (59), polarized segregation, has been offered as the agent of non-reciprocal fertility in the *Culex pipiens* complex, and it also has been discounted (23). McClelland (59) suggests that the different crossing groups carry respective pairs of dominant or recessive alleles in their nuclear genetic constitution and these alleles condition the cytoplasm so that the interrelation of alleles and cytoplasm would react favorably or unfavorably in the development of larvae. A system of dominance and recessiveness would prevail with respect to alleles and allelic combinations. Two alleles might show equal dominance with respect to other alleles and these two alleles would be lethal in each other's cytoplasm. Introduction of a dominant allele into a different dominant allelic conditioned cytoplasm would be lethal. A combination of a dominant and a recessive pair of alleles would condition the cytoplasm to a type representative of the dominant member of the pair. Introduction of a recessive allele into a dominant conditioned cytoplasm is viable in most cases. An essential aspect of this hypothesis is a preferential type of segregation present in certain males, but not necessarily in the females. This preferential type of segregation would occur in certain combinations of a heterozygous dominant and recessive pair of alleles and would selectively eliminate paternal chromosomes carrying the recessive allele.

Assigning letters for clarification: the dominant Ha strain would be \( M,M \) with a \( M \) conditioned cytoplasm, the dominant Pa strain would be \( N,N \) with a \( N \) conditioned cytoplasm, and the heterozygous HC could be \( N,F \) with a \( N \) conditioned cytoplasm. The Paris \( N \) alleles would be incompatible in the Hamburg \( M \) conditioned cytoplasm. HC alleles would be
partially compatible in the Hamburg M conditioned cytoplasms. The r alleles would be compatible, but the N alleles would be incompatible. Both alleles of the HC strain would be compatible in the N conditioned cytoplasm of the Pa strain. In order to explain the incompatibility of Cross V, the HC strain would have to show the male meiotic drive mechanism of selection against the recessive r allele. Without this selection, the recessive r allele would appear in the F₁ hybrid of this cross. The presence of this allele would then increase the compatibility of Cross V. If polarized segregation occurred in Cross I involving Pa females and HC males, then it should also have been in operation when Ha females were bred by HC males, and the 32.1% average hatch rate would have been decreased to an almost 0.0% average hatch rate. Cross VI should also have shown polarized segregation and a near zero average hatch rate, not the 27.5% average hatch rate which occurred. Polarized segregation could not be a factor in the cytoplasmic inheritance shown by the crosses of this investigation.

The crosses in Fig. 1 were not designed to investigate various proposed causative agents of cytoplasmic inheritance in the Culex pipiens complex. The design was primarily to permit a genetic analysis of any mitochondrial structural protein differences observed by polyacrylamide gel electrophoresis. Gel differences in the structural proteins of the Pa and HC strains should also have been shown as gel differences in the F₁ hybrids resulting from Cross I and Cross II. The two F₁ hybrids are identical in every respect, except for their maternally inherited cytoplasm. The hybrids should and do show the incompatibility and partial incompatibility of their maternal parents.
The statistical difference in the results shown by Cross IV and Cross VI (Fig. 1) may have been due to the males selected for use in Cross VI. F₁ animals resulting from Cross II were mass reared and as the adults began to emerge, males were collected and used in the cross. These males probably came from an egg raft of a female whose male progeny would have shown a lower than average hatch rate when bred with Ha females. A second possible cause for the statistical difference in these two crosses may be the result of selection. It has been previously shown that HC cytoplasm is probably a combination of two cytoplasmic factors. One member of this combination is considered to be the same factor present in the Pa cytoplasm and, therefore, compatible to that cytoplasm. The other factor is considered to be incompatible to the Pa cytoplasm, resulting in a natural selection against this second factor. F₁ animals resulting from Cross I would possess essentially the same cytoplasmic inheritance factor as their Pa maternal parent. The reciprocal cross, Cross II, could result in a slightly changed F₁ cytoplasm. The HC cytoplasm contains a large quantity of the Pa cytoplasmic factor and would, therefore, show no selection against the introduction of an additional small quantity of this same factor. This small addition of the Pa cytoplasmic factor could be responsible for the small statistical change noted between the results of Cross IV and Cross VI. The statistical P value of 0.04 suggests the possibility of a dilution factor. Progressive changes in the cytoplasmic inheritance system of the Culex pipiens complex have been shown by this investigation.
Several statements made by Laven concerning criteria of cytoplasmic incompatibility inheritance in the *Culex pipiens* complex are not in accord with the findings of this investigation. One of these is the "mutation" of a given cytoplasmic type to a clear cut different type in a single step (43). The results shown in Table III do not support this assertion. They do, however, indicate a stepwise change from a partial incompatibility to an almost complete incompatibility, and are in accord with the findings of French (26) while working in Laven's laboratory. This change has taken place over a period of approximately two years. During this period of time each strain was bred and selected for high percentage of hatch. The stepwise change in the incompatibility of Pa males times Ha females is a result of intra-strain selection. A stepwise movement to a new cytoplasmic type will alter the hypothesis of speciation by cytoplasmic inheritance (41). Rather than an immediate mutation to a new type, the evolution, in nature, to a new type would be a progressive dynamic process. This alteration significantly alters the overall hypothesis of speciation by cytoplasmic inheritance.

A hypothetical lack of intermediate situations seems to be a fundamental aspect of Laven's *Speciation by Cytoplasmic Isolation in the Culex pipiens Complex* (41). On this assumption, he indicates that only three kinds of occurring mutations would seem possible: mysterious or hidden changes which do not immediately affect the crossing abilities of two closely associated populations, unilateral changes which would result in incompatibility in one direction, and bilateral changes which
would be the result of separate changes at different places affecting the crossing ability in both directions.

The fact that stepwise changes do occur, in conjunction with the knowledge that intermediate conditions are known (6, 17, 18, 23), must lead to a new evaluation of the concept of speciation by cytoplasmic inheritance in the Culex pipiens complex. Changes to a unilateral or bilateral incompatibility as an immediate effect are not comprehensible. These two forms of incompatibility could only result as a consequence of long periods of natural selection for either of these conditions in a natural population. Heterogeneity in the cytoplasmic inheritance system of a natural population of these organisms has been shown (22, 23). This heterogeneity was expressed as a partial incompatibility and the suggestion was made that its occurrence is more widespread in nature than had previously been supposed. Many lethals, therefore, may originate as a partial incompatibility of the cytoplasm and would result in an interruption of gene flow within the natural population. This mechanism could be a potent force in the evolutionary process.

Another aspect of cytoplasmic inheritance discussed by Laven as a characteristic is, "the penetration of the sperm stimulates the egg to meiosis and embryonic development which runs to an advanced stage; the sperm itself is blocked from karyogamy and does not contribute anything more; the organogenesis is irregular and distorted." (45). In accord with the results of French (23), irregular and distorted unhatched larvae as a result of fertilization were observed (Table II). These larvae had undergone karyogamy as evidenced by the presence of the normal eye color in these unhatched distorted individuals. Unhatched
eggs which had undergone gynogenetic development showed the white eyes of their maternal parent, while unhatched eggs with wild or normal colored eyes were the result of fertilization.

The basic crosses made in this investigation have shown that at least three different cytoplasmic factors or plasmagenes are present in the four strains of mosquitoes. One factor present in the Pa strain was compatible in the HC strain, but not in the Ha strain. A second factor, which may or may not have been in conjunction with the first factor, present in the HC strain, was compatible in the Pa strain and partially incompatible in the Ha strain. The third factor involved the incompatibility of the three strains in the Bl cytoplasm.

Results of the basic crosses of Fig. 1 and Fig. 2 can be explained in terms of polymorphic forms of mitochondria. Polymorphic forms of mitochondria have been shown and these different forms show strong interactions when mixed in vitro (24). Mitochondria isolated from several cytoplasmic mating types were mixed in various proportions. These mixtures were then tested for the specific activity of cytochrome C oxidase and other mitochondrial enzymes. If mitochondria from the strains tested had been all of the same form, then mixing of these mitochondria in different proportions would have shown no increase or decrease in the activity of mitochondrial enzymes. There were changes in the activity with respect to the proportions of various mitochondria used. Purified mitochondria from three different mating types were mixed in vitro in various ratios and tested for activity. Highly significant differences were found when the mitochondria were mixed in different proportions. These highly significant differences were reflected in
all six mitochondrial enzyme systems assayed. Different combinations resulted in both depressed and highly elevated enzymatic activity when the various mixtures were compared with the parental mitochondrial types. Mitochondria, therefore, are polymorphic and show an interaction when these various forms are placed in close proximity to each other as has been shown in wheat and corn by Sarkissian, et. al. (60, 82, 83). The cytoplasmic factors or plasmagenes, spoken of previously, can now be referred to as mitochondrial forms which interact under appropriate experimental conditions.

A mitochondrial form present in the Pa cytoplasm would react with a different mitochondrial form present in the Ha cytoplasm. This reaction would be unfavorable if small quantities of the Pa mitochondrial form were mixed with large quantities of the Ha mitochondrial form, as would be expected in fertilization. The HC cytoplasm would be polymorphic with respect to its mitochondrial form. One type of mitochondrial form would be compatible with the mitochondrial form of the Pa cytoplasm and incompatible with mitochondria of the Ha cytoplasm, while the other mitochondrial form of the HC strain would be incompatible in the Pa cytoplasm and compatible in the Ha cytoplasm. All mitochondrial forms present in the Pa, Ha, and HC strains interact unfavorably when small quantities of these forms are introduced into the B1 cytoplasm with its different mitochondrial form. There are then at least three different mitochondrial forms present in the involved strains and these forms could be in varying proportions within each strain.
Sperm are a limited subsample of the paternal germinal cytoplasm (23). If meiotic division of a spermatogonial cell results in sub­samples of that spermatogonial cell's cytoplasm, then we should also expect this same sampling to occur during mitotic division of somatic cells. Each somatic cell should be a limited subsample of the original cytoplasm of the zygote. A zygote then might contain two forms of mitochondria whose ratio was such that a beginning of the developmental process is permitted or even in some cases enhanced. As mitotic division occurs, certain cells may arise which possess the exact ratio of mitochondrial forms and depression of mitochondrial enzyme activity of those cells would result. These cells would be depressed in their activity with respect to other cells. This process could continue to such an extent that the resulting unhatched larvae would show irregular development of many structures. One of these structures is the eyes (25).

Based on the work of others (20, 21, 65), Ashwell and Work (4) hypothesize that "assuming that mitochondrial DNA codes specifically for certain mitochondrial proteins, mutation in mitochondrial DNA should result either in alteration or in disappearance of these proteins."

Mitochondria originate from pre-existing mitochondria (34, 49, 55, 57, 95). Purified mitochondrial structural proteins account for approximately 75% of total radioactivity incorporated by mitochondria (36, 77). Mitochondrial structural protein is coded for and synthesized within the mitochondria (36). Changes in the amino acid composition of mitochondrial structural protein in one organism have been reported (98). Mitochondrial complementation (24) has shown that mitochondria extracted
from three distinct cytoplasmic mating types are different. All these facts when considered in toto support the initial assumption that there should be a variation in the mitochondrial structural protein of cytoplasmically unique strains of the Culex pipiens complex.

One possible reason for the negative results obtained by polyacrylamide gel electrophoresis could very well be amino acid substitution within the structural proteins, so as to affect the organization and/or assembly of the respiratory enzymes. The differences in amino acid composition of mitochondrial structural proteins result in altered enzyme activity of enzymes bound to these membranes (98). This type of change could result in a protein which is different in function, but similar in electrophoretic mobility.

A preferable suggested biological phenomenon (29) which might elicit a phenotypic change, but one which would be undetectable in the mitochondrial structural proteins using gel electrophoresis, is in allosteric regulation. The term allosteric (50) literally means "other-space" or "other site." Allosteric proteins can possess at least two sites; one, a binding or catalytic site, and the other a modulator site. An allosteric effector, binding at the modulator site, may inhibit or stimulate a reaction at a given substrate concentration by exerting an influence on the catalytic site. Conformational proteins (29) differ in their ability to enhance or depress the enzyme efficiency of a particular mitochondrial species. Penrose et. al. (72) working with a leucine-binding protein from E. coli were able to show that reversible conformational changes did not affect the electrophoretic properties of the involved protein.
Results of this investigation have shown that polyacrylamide gel electrophoresis is not amenable to the detection of mitochondrial structural protein differences of cytoplasmically unique strains of the *Culex pipiens* complex. Detection of these mitochondrial structural proteins will of necessity require a more precise genetic or biochemical tool. Amino acid analysis is one approach which would no doubt resolve minor differences in the mitochondrial structural proteins of these cytoplasmically different mosquitoes. This technique (98) has been used previously in a study of cytoplasmically different strains of *Neurospora* and the results have shown that the different strains possess different amino acid compositions. A second possible approach to the study of mitochondrial structural protein differences is hybridization. Hybridization of purified mitochondrial structural proteins with various mitochondrial fractions from other strains could be expected to resolve differences in mitochondrial structural proteins.

Studies in the cytoplasmic inheritance system of the *Culex pipiens* complex are important for this is the only known system of its kind among the many higher eucaryotic animals. Similar systems of cytoplasmic inheritance in other higher animals have been proposed, but none has the phenotypic manifestations which are required in scientific investigation.

One very important aspect of this cytoplasmic inheritance system is in genetic control of insect pests and an alternative to the use of insecticides. Another importance is that these organisms serve as a model system for studying the basic phenomenon of cytoplasmic inheritance in man and other higher animals.
When crosses are made involving cytoplasmic partially incompatible strains of *Culex*, a study of unhatched eggs reveals that the following structures are distorted in inviable embryos: thorax, somites, egg tooth, mouth parts, and eyes. Of these, the structure most easily studied is the eye (23). The degree of abnormality varies within any given egg raft from almost complete eye development to scattered patches of pigment cells throughout the length of the egg.

Purified mitochondria (24), from different strains of the *Culex pipiens* complex, when combined in various proportions show complementation, an alteration in the efficiency of the electron transport and oxidative phosphorylation systems. These two systems are ultimately responsible for the production of ATP, which is required in the metabolic process of all living cells. Different proportions of mitochondria result in significant differences of enzymatic activity of those mitochondria.

Offspring resulting from crosses involving certain strains of the *Culex pipiens* complex die as unhatched larvae. This death, during the embryonic stage, is a result of highly irregular and disproportionate cell growth and tumor formation. These patterns evidently result when interaction occurs within embryonic cells which contain different molecular forms of mitochondria. As has been stated, *in vitro* interaction of different molecular forms of mitochondria results in gross differences in the enzymatic activity of the involved mitochondria. These differences are expressed in an *in vivo* teratological growth and extensive tumor formation. The aberrant growth would result when cells contain various proportions of several forms of complementing mitochondria (29).
In man some aberrant conditions of the mentally retarded occur during embryonic development. These existing conditions can be understood from the basic information obtained from the study of the cytoplasmic incompatibility inheritance system of the *Culex pipiens* complex. The literature is replete with information concerning the large number of congenital abnormalities found in retardates. This is another form of terratological development. The number and severity of these aberrations varies with each individual.

There are discrepancies in the expected association of karyotype to syndrome in many mentally retarded individuals. Some apparently normal individuals show an abnormal karyotype (14, 54, 71, 74, 88, 89, 97) which is usually found in a specific syndrome and some mentally retarded persons with specific syndromes possess a normal karyotype (1, 9, 11, 52, 61, 70, 90, 91). These facts indicate that a chromosomal aberration is not the final answer as to all causative factors of mental retardation. Malformation of the mentally retarded which occurs during human embryonic development has a parallel in the cytoplasmic incompatibility inheritance system of the *Culex pipiens* complex.

In this model system in *Culex* mosquitoes, malformations occurring in eggs apparently result from interactions between different forms of mitochondria. Abnormalities associated with some mentally retarded children apparently arise during embryonic development as a cell energy disequilibrium.

Genetic investigations into the cytoplasmic inheritance system of the *Culex pipiens* complex are important in basic research, as well as in its many far reaching implications, especially in man.
Table I. Intrastrain egg counts and percentage of hatch.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>NUMBER OF EGG RAFTS</th>
<th>LARVAE</th>
<th>UNEMBRYONATED</th>
<th>EMBRYONATED, UNHATCHED</th>
<th>EMBRYONATED, EYE DISTURBION</th>
<th>TOTAL EGGS</th>
<th>AVERAGE HATCH PERCENTAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pa</td>
<td>12</td>
<td>1002</td>
<td>32</td>
<td>4</td>
<td>2</td>
<td>1040</td>
<td>88.9</td>
</tr>
<tr>
<td>HC</td>
<td>30</td>
<td>2257</td>
<td>364</td>
<td>137</td>
<td>53</td>
<td>2811</td>
<td>79.2</td>
</tr>
<tr>
<td>Ha</td>
<td>11</td>
<td>1239</td>
<td>317</td>
<td>64</td>
<td>32</td>
<td>1652</td>
<td>75.2</td>
</tr>
<tr>
<td>Bl</td>
<td>17</td>
<td>1431</td>
<td>338</td>
<td>6</td>
<td>3</td>
<td>1778</td>
<td>84.0</td>
</tr>
</tbody>
</table>
Table II. Egg counts and percentage of hatch for the basic crosses and test crosses involving the Pa, HC and Ha strains.

<table>
<thead>
<tr>
<th>CROSS</th>
<th>NUMBER OF EGG RAFTS</th>
<th>LARVAE</th>
<th>UNEMBRYONATED</th>
<th>EMBRYONATED, UNHATCHED</th>
<th>EMBRYONATED, CYNOGENETIC</th>
<th>EMBRYONATED, EYE DISTORTION</th>
<th>TOTAL EGGS</th>
<th>AVERAGE HATCH PERCENTAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Pa x HC</td>
<td>34</td>
<td>2033</td>
<td>758</td>
<td>54</td>
<td>0</td>
<td>34</td>
<td>2879</td>
<td>71.6</td>
</tr>
<tr>
<td>II. HC x Pa</td>
<td>23</td>
<td>2224</td>
<td>263</td>
<td>49</td>
<td>0</td>
<td>27</td>
<td>2563</td>
<td>88.3</td>
</tr>
<tr>
<td>III. Ha x Pa</td>
<td>30</td>
<td>56</td>
<td>3036</td>
<td>78</td>
<td>9</td>
<td>1109</td>
<td>4288</td>
<td>1.3</td>
</tr>
<tr>
<td>IV. Ha x HC</td>
<td>22</td>
<td>865</td>
<td>1845</td>
<td>49</td>
<td>12</td>
<td>104</td>
<td>2875</td>
<td>32.0</td>
</tr>
<tr>
<td>V. Ha x (PaHC)</td>
<td>16</td>
<td>34</td>
<td>1739</td>
<td>48</td>
<td>7</td>
<td>584</td>
<td>2413</td>
<td>1.4</td>
</tr>
<tr>
<td>VI. Ha x (HCPa)</td>
<td>11</td>
<td>481</td>
<td>1168</td>
<td>21</td>
<td>9</td>
<td>57</td>
<td>1736</td>
<td>27.5</td>
</tr>
</tbody>
</table>

Note: Females are indicated before the cross sign and males following the cross sign.
Table III. Crosses involving Ha females and Pa males made from 1970 through 1971.

<table>
<thead>
<tr>
<th>CROSS PERIOD</th>
<th>NUMBER OF EGG RAFTS</th>
<th>LARVAE</th>
<th>UNEMBRYONATED</th>
<th>EMBRYONATED, UNHATCHED</th>
<th>EMBRYONATED, EYE DISTORTION</th>
<th>TOTAL EGGS</th>
<th>AVERAGE HATCH PERCENTAGE</th>
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</thead>
<tbody>
<tr>
<td>Spring 1970</td>
<td>6</td>
<td>244</td>
<td>426</td>
<td>12</td>
<td>41</td>
<td>723</td>
<td>32.0</td>
</tr>
<tr>
<td>Summer 1970</td>
<td>18</td>
<td>613</td>
<td>1486</td>
<td>102</td>
<td>166</td>
<td>2367</td>
<td>20.4</td>
</tr>
<tr>
<td>Fall 1970</td>
<td>27</td>
<td>120</td>
<td>1386</td>
<td>18</td>
<td>268</td>
<td>1792</td>
<td>5.7</td>
</tr>
<tr>
<td>Summer 1971</td>
<td>30</td>
<td>56</td>
<td>3036</td>
<td>78</td>
<td>1118</td>
<td>4451</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Table IV. Egg counts and percentage of hatch for the crosses and backcrosses of Pa, HC and Ha strains into the Bl cytoplasm.

<table>
<thead>
<tr>
<th>CROSS</th>
<th>NUMBER OF EGG RAFTS</th>
<th>LARVAE</th>
<th>UNEMBRYONATED</th>
<th>EMBRYONATED, UNHATCHED</th>
<th>EMBRYONATED, EYE DISTORTION</th>
<th>TOTAL EGGS</th>
<th>AVERAGE HATCH PERCENTAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bl x Ha</td>
<td>20</td>
<td>0</td>
<td>958</td>
<td>771</td>
<td>1040</td>
<td>2769</td>
<td>0.0</td>
</tr>
<tr>
<td>Bl x Pa</td>
<td>26</td>
<td>13</td>
<td>1658</td>
<td>674</td>
<td>1118</td>
<td>3463</td>
<td>0.3</td>
</tr>
<tr>
<td>Bl x HC</td>
<td>43</td>
<td>8</td>
<td>3430</td>
<td>109</td>
<td>2664</td>
<td>6211</td>
<td>0.1</td>
</tr>
<tr>
<td>Bl x (HaBl)</td>
<td>5</td>
<td>0</td>
<td>304</td>
<td>191</td>
<td>218</td>
<td>713</td>
<td>0.0</td>
</tr>
<tr>
<td>Bl x (PaBl)</td>
<td>6</td>
<td>0</td>
<td>450</td>
<td>204</td>
<td>239</td>
<td>893</td>
<td>0.0</td>
</tr>
<tr>
<td>Bl x (HCBl)</td>
<td>5</td>
<td>1</td>
<td>482</td>
<td>9</td>
<td>411</td>
<td>902</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Note: Females are indicated before the cross sign and males following the cross sign.
Plate 1. Electropherogram of mitochondrial structural proteins of the *Culex pipiens* complex

1. Isolated mitochondrial structural protein - 50 λ application

2. Purified mitochondria structural protein - 50 λ application

3. Purified mitochondrial structural protein with performic acid treatment - 50 λ application
Plate 2. Electropherogram of purified mitochondrial structural protein with performic acid treatment.

1. Pa - 100 λ application
2. HC - 100 λ application
3. Ha - 100 λ application
4. Bl - 100 λ application


27. French, W. L. Indications of cytoplasmic incompatibility within a panmictic population of Culex quinquifaciatus. (Unpublished)


29. French, W. L. Mitochondrial complementation, disproportionate cell proliferation and a new concept and approach to cancer induction, suppression and control. (Unpublished)


VITA

Charles C. Faust III was born in Alexandria, Louisiana on January 3, 1925. He attended school for eleven years in the Lake Charles City School System, and was graduated from Lake Charles High School in 1943. In that year, he volunteered for service in the United States Army Air Corps and spent the next thirteen months in cadet flight training, graduating from pilot school as a Second Lieutenant. The next two years were spent instructing cadets, flying B-29 missions over Japan, and engaging in occupation duty on Okinawa. Charles is now retired from the United States Air Force Reserve with the rank of Lieutenant Colonel.

He entered John McNeese Junior College upon separation from the service, and two years later transferred to the Baton Rouge campus of Louisiana State University. There he completed an undergraduate program of Science Education in 1950. He has seventeen years experience teaching in the secondary school system of Louisiana, and five years experience at the college level. Requirements for a Master's degree in Education were completed in 1956.

Charles and his wife, the former Elizabeth Stark, have two children; Candace Lynn Hyde and Charles Carroll Faust IV.
EXAMINATION AND THESIS REPORT

Candidate: Charles C. Faust, III

Major Field: Zoology

Title of Thesis: Studies on the Cytoplasmic Incompatibility System in Culex pipiens: A Genetic and Electrophoretic Study of Mitochondrial Structural Proteins

Approved:

[Signatures]

Major Professor and Chairman

[Signature]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination: 5 November 1971