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The Effects of Las on the Ultrastructure of the Teleost Chorion

Mark Xavier VanCura
Loyola University Chicago

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THE EFFECTS OF LAS ON THE ULTRASTRUCTURE OF THE TELEOST CHORION

by

Mark Xavier VanCura

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

June

1976
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VITA

Date and Place of Birth: November 3, 1952; Berwyn, Ill.


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INTRODUCTION

The teleost chorion serves as a natural barrier to environmental agents. Although there have been studies of the permeability of the chorion (Manner and Muehleman, 1975), little attention has been devoted to either the chorionic structure or ultrastructure. Investigators in the field of aquatic pollution have not, in general, considered the teleost chorion as an important parameter in their toxicity studies. Manner and Thompson (1974) have postulated that the different response of embryos to these pollutants at various stages of embryogenesis is the result of a different susceptibility of embryonic tissues. Although this may be true, it may also be possible that the chorion, acting as a barrier between the embryo and its aquatic environment, might allow different amounts of pollutants to pass into the embryo at specific stages of embryogenesis. Manner and Muehleman (1975) supported this hypothesis by demonstrating that the chorion surrounding the fathead minnow (Pimephales promelas Rafinesque) embryo becomes more permeable as embryogenesis progresses. This change in permeability necessarily dictates that the age of a developing embryo must be considered an important parameter in all
aquatic toxicity and teratogenicity research.

The chorion has been described using both the light and the electron microscopes. Hisaoka (1958), using the zebra fish Brachydanio rerio, investigated the chorion using light microscopy. He described it as a tough, fibrous leakage membrane, with an average thickness of 10 microns. He also pictured it as a sieve-like membrane containing many small openings or pores. He concluded that the chorion served both as a leakage and protective membrane for the developing embryo. Yamamoto and Yamagami (1975) described the chorion of the Japanese Medaka (Oryzias latipes) and studied the morphological changes in the chorion caused by the hatching enzyme.

The fish usually used in toxicity studies have been the zebra fish (Brachydanio rerio); the Japanese Medaka (Oryzias latipes); and the fathead minnow (Pimephales promelas). The fathead minnow is indigenous to the United States and it is used quite extensively by the Environmental Protection Agency. It is for this reason that the fathead minnow was used in this research.

The primary purpose of this research is to study the ultrastructure of the chorion of the fathead minnow. One of the pollutants currently under investigation in many laboratories is linear benzene sulfonate (LAS), a component of biodegradable detergents. It has already been found to be teratogenic as well as toxic to teleosts (Manner and
Dewese, 1973). LAS is the most commonly used surfactant in the detergents presently in use in the United States. It is quite similar to the surfactants used in previous years except that LAS is biodegradable, i.e., readily broken down by the action of bacteria present in the environment.

LAS is a surface acting agent (Stoker and Seager, 1972). This quality suggests that it might have the capability of altering the structure of the chorion and thereby changing its diffusion properties. Therefore, the secondary purpose of this thesis is to determine whether or not LAS has an effect on the ultrastructure of the chorion of the fathead minnow.
REVIEW OF THE LITERATURE

The Fathead Minnow

The embryonic development of those teleosts that are used most frequently in laboratory studies are well documented. The developmental stages of the zebrafish, *Brachydanio rerio*, have been reported by Hisaoka (1960). The Japanese Medaka, *Oryzias latipes*, has been described by Briggs and Egami (1959). These fish were not found to be satisfactory for aquatic pollutant studies, for none of them are found in inland bodies of water within the continental United States.

The fathead minnow, *Pimephales promelas*, has been established by many laboratories as an assay fish for the determination of the toxicity of aquatic pollutants, (Bender, 1969; Mount and Stephan 1969; Arthur and Eaton, 1971). The early embryology of the fathead minnow was described recently by Manner and Dewese, (1974). Most of the results obtained on the fathead minnow embryos were reported as either the percent of fish hatching, or in terms of the median tolerance limit, (TLm), of the embryos with regard to the toxicant being used. The fathead minnow was also used in permeability
studies by Manner and Muehleman (1975), who in their work suggested the possibility of an altered chorionic structure during embryogenesis.

The peak spawning of the fathead minnow, under normal environmental conditions, occurs during the month of July. The males of the species are larger than the females and around the time of breeding the males become darker in color (Carlson, 1967). The breeding and early embryology of the fathead minnow has been documented by Manner and Dewese (1974).

The Teleost Chorion

The fertilized egg of the fathead minnow is approximately 1.0 mm. in diameter and until hatching it is surrounded by a clear chorion. It is the chorion that seems to act as a protective membrane for the developing embryo. The structure of the chorion has been described using both the light and the electron microscopes. Using the light microscope, Hisaoka (1958), described the chorion of the zebra fish, Brachydanio rerio. He pictured the chorion as a sieve-like membrane having an average thickness of 10 microns.

The ultrastructure of the teleost egg and the changes following fertilization are much less well known (Hagstrom and Lonning, 1968). Electron microscope studies of the chorion have shown it to consist of an outer, more electron
dense layer and an inner, less electron dense layer, often multilamellar layer. The development of these two layers as well as their structure is well documented (Gotting, 1966 and Anderson, 1967). However, many questions are still unsolved.

The ultrastructure of the chorion of the cod, Gadus morrhua, has been described by Lonning (1972). The chorion of this species is approximately 4.5 microns thick. The inner part of the chorion presents 5 lamellar layers. The outer part of the chorion presents 3 more lamellar layers. The thickness of the lamellae decreases in an outward direction, with the most inward lamellae being 1 micron thick and the most outward lamellae being 0.4 microns thick.

The chorion of the ripe egg of Limanda limanda has been described using electron microscopy by Hagstrom and Lonning (1968). They described the chorion as being 2.5 microns thick and consisting of 9 lamellar layers of equal thickness. They also described an outer homogenous layer that is interrupted by pores. These pores often widen where they reach the lamellar part and demonstrate a type of network within the lamellae.

Yamamoto and Yamagami (1975) described the chorion of the Japanese Medaka, Oryzias latipes. Their study showed that the chorion consists of a thin outer layer and a thick, multilamellar inner layer. These same general structural features have been observed in the Medaka with the light
microscope, (Nakano, 1956; Ogawa and Ohi, 1968), and in the
cod with electron microscopy (Lonning, 1972). Yamamoto and
Yamagami (1974) suggested that the outer layer is composed
of a sheet of electron dense lamina and a fibrous coat, and
that the inner layer consists of about twelve electron
dense lamellae separated by about eleven interlamellar
portions of lower electron density. Their work also showed
that the outer surface of the chorion is found to be covered
by a somewhat irregular, honeycomb like pattern, as was first
reported by Kamito (1928) using light microscopy, while the
inner surface showed a pattern of more or less wavy lines.

The chorion seems to serve as a protective mem-
brane for the developing embryo. The teleost chorion, as
described by Hisaoka (1958) for the zebra fish, has been
termed a "leakage membrane". According to Hisaoka (1958),
although the chorion is tough and fibrous, water and electro-
lytes can readily penetrate through the chorion because of
the relatively large diameter of the pores which penetrate
through the chorion. Using phase- contrast microscopy,
Hisaoka found the pores to have an average diameter of 1.5
microns. Hagstrom and Lonning (1968) suggested a change in
these pores after fertilization. In Platichthys flesus
embryos Hagstrom and Lonning (1968) noticed a marked change
in the surface appearance of the outer layer of the chorion
and noticed that all layers of the lamellar part of the chor-
ion looked different three to five days post- fertilization.
Chorions protect not only the eggs of many species of fish, but also the eggs of some insects (Chauvin et al., 1974). The dragon fly egg is surrounded by three membranes, an innermost vitelline membrane and two outer chorionic layers (Kawasaki et al., 1974).

Hagenmaier (1974) isolated a chorionic enzyme from the teleost *Salmo gairdneri*. This chorionase was isolated specifically from the hatching fluid of the embryo and it is responsible for the initiation of the hatching process. Yamamoto and Yamagami (1975) studied the morphological changes in the chorion of the teleost, *Oryzias latipes*, caused by the hatching enzyme. It was found that the enzyme digested the inner layers of the chorion while the outer layer remained undigested. They showed that after treatment with the hatching enzyme, the surface of the outer chorionic layer became rougher, while the inner layer underwent a gradual reduction in the number of lamellae progressing from the inner to the outer surface until it was completely digested.

A characteristic of the teleost chorion is the micropyle, a pore that allows the passage of sperm and therefore fertilization. At the animal pole, the egg membrane is pierced by the micropylar canal. Following insemination, the sperm are located in a single row in the micropylar canal. When the first sperm reaches the egg surface, the other sperm are pushed out of the canal (Yamamoto, 1952).
In *Limanda* eggs, Yamamoto (1952) observed the formation of a pluglike deposit at the entrance of the micropyle immediately following fertilization. According to Sakai (1961) the discharge of extra sperm is accomplished by the secretion of colloidal material into the canal from the inside. This material occasionally bulges out from the canal, forming a plug. Ginsberg (1961) showed that the mechanism responsible for monospermic fertilization in fishes seems to be attributable to the fact that a fertilizing sperm can reach the egg surface only through the micropyle, which undergoes some marked changes, making it impermeable to supernumerary sperm following fertilization.

**Biochemical Nature of the Chorion**

Many studies concerning the chemical composition of the chorion have been done, Young and Inman (1938) studied the protein of the chorion of salmon eggs, *Salmo salar*. Their research into the chemical nature of the chorion stemmed from an interest in the softening and rupture of the chorion that occurs during hatching. Young and Inman (1938) suggested that the softening of the chorion is the result of some structural change, and believed it to be of significance in the semipermeability of the chorion. They concluded that the chorion was basically protein in nature, resembling a keratin.

Hagenmaier (1973) did an extensive study on the
structure, polysaccharide and protein cytochemistry of the chorion of the trout egg, *Salmo gairdneri*. His interest in the structure of the chorion resulted in the close relationship he saw between the process of hatching and the chemical composition of the chorion. Using cytochemical methods, he described the biochemical constitution of the chorion, and using electron microscopy showed the ultrastructure of the chorion. He suggested that there are two layers that are discernable using cytochemical tests. The outer layer contains protein, neutral carbohydrates, and mucopolysaccharides. The inner layer consists entirely of protein.

Costello, et al (1957) and Austin (1961) suggested that the chorion may be of a mucopolysaccharide or mucoprotein nature. Yamamoto and Yamagami (1975) suggested that the chorion of the Medaka, *Oryzias latipes*, is composed of a polypeptide-mucopolysaccharide complex. Young and Inman (1938) estimated the amino acid content of the salmon chorion. They found a total of eighteen amino acids with a low content of cystine.

**Biochemical Analysis of LAS**

LAS, linear alkyl benzene sulfonate, is a component of biodegradable detergents. LAS behaves as a surfactant, lowering the water surface tension. The surfactant contains a nonpolar end which is hydrophobic and a polar end which
is hydrophilic.

When a substrate is cleansed, an adsorption of surface active molecules takes place at the interface so that dirt particles are lifted from the substrate. The surfactant then adsorbs these particles, the hydrophilic end keeping the particles in suspension (Rosen, 1972).

Of the components of detergents, it is the surfactant portion which has caused the greatest concern during the past twenty years. Alkyl benzene sulfonate, ABS, was the most widely used detergent surfactant until the 1960's. By 1962, ABS accounted for 70% of the surfactant volume of detergents and it was found that the foaming in waterways was caused by the residues from ABS (Brenner, 1969). Because of its highly branched alkyl structure, this surfactant was found to be very resistant to biodegradation. Consequently, a surfactant that showed a more rapidly degradable, straighter alkyl chain came forth as an ABS replacement. This more modern surfactant is linear alkyl benzene sulfonate, LAS, and it has been considered to be biologically safer because of its biodegradable properties. This biodegradation has been proposed to occur as a result of the oxidation of the terminal methyl group to a carboxylate followed by degradation continuation via beta oxidation. The straighter the alkyl chain, the faster the degradation (Swisher, 1963; Hammerton, 1955).
A property of surfactants related to their detergent property is their tendency, in aqueous media, to aggregate into groups called micelles (Swisher, 1970). At low concentration, such as one part per million, these surfactants are present as individual molecules randomly distributed along the interfacial surface of air and water. However, when the surfactant concentration is increased, it eventually reaches the critical micelle concentration, a concentration at which micelles are formed (Rosen, 1972). These micelles occur at concentrations of at least one hundred parts per million, and studies have shown that significant cleansing power or detergency is only reached at this critical micelle concentration (Schwartz, 1971).

The length of the alkyl chain in surfactants can vary between eight and eighteen carbons, but the majority of the molecules have lengths between ten and fourteen (Swisher, 1963). A commercial preparation is not composed of a uniform surfactant; it is a mixture of compounds with varying alkyl lengths as well as varying phenyl and sulfonate positions. The benzene ring is normally attached to a secondary position and the sulfonate can be in meta or para position, but seldom in ortho, because of steric hindrance (Swisher, 1963). The best detergency is obtained from an LAS with an average 12.5 carbon chain length (Sweeney and Olson, 1964).

Swisher (1970) gave possible degradation routes
for the benzene ring of LAS. He stated that catechol is a probable intermediate of most routes. This catechol is formed in an enzyme-catalyzed oxidation step. The next step would be the splitting of the ring between either of the two hydroxyl groups; then betaketoacidic acid is formed which is then split further to acetate and succinate via beta-oxidation. If the split occurs adjacent to the hydroxyl groups, this path leads to the formation of formic acid, acetaldehyde and pyruvic acid.

Investigations into pollutant toxicity are quite extensive and several methods have been developed to measure this toxicity to fish (Sprague, 1970).

The first studies on the effects of LAS, when administered orally to mammals, were by Kay, et al (1965). They found that LAS caused no adverse effects when fed to rats in concentrations of two hundred, one thousand, and five thousand parts per million for ninety days.

Numerous experiments have shown LAS to be quite toxic to several species of fish and fish embryos (Cairns and Scheier, 1962; Manner and Dewese, 1973; Thatcher and Santer, 1967).

Willis (1954) has suggested that detergent surfactants may play a role in the inhibition of certain enzymes. Therefore, the failure of some experimental fish to hatch may possibly be attributed to the inhibition of the hatching enzyme.
In a report by Hokanson and Smith (1971) it was shown that the most important environmental factors influencing the toxicity of LAS to the bluegill were dissolved oxygen, water hardness, and acclimation to LAS. The most sensitive stage of the developing bluegill to LAS was the feeding sac-fry, while the most tolerant stage was the newly hatched sac-fry. This illustrates the importance of age consideration in detergent toxicity experimentation.

In a review paper by Abel (1974) it was reported that the acute toxic effects of detergents on fish include gill damage, destruction of chemoreceptor organs and epidermis and pharyngeal wall damage. It was also proposed in this paper that detergents probably cause denaturation of proteins and the alteration of membrane permeability and transport characteristics.
MATERIALS AND METHODS

Breeding

Adult fathead minnows, *Pimephales promelas* were placed in breeding tanks provided with dechlorinated tap water and maintained at 23°C ± 1°C. The water was aerated and conditioned by an in-tank aeration system with activated charcoal and glass wool filtration. The adult fish were conditioned for breeding by being subjected to a sixteen hour photo period. They were fed daily with brine shrimp, tubifex and a vegetable conditioning food. Inverted halves of five inch diameter clay and asbestos pipes were placed on the bottom of the tanks. Fertilized eggs were collected from the underside of the pipe-halves.

Staging

Approximately one hundred normal fathead minnow embryos were selected at five different stages. These were to be used to study the ultrastructure of the normal chorion. An additional one hundred fathead minnow embryos were selected at the five different stages to be treated with LAS. The five different stages were Stage F, day of fertilization; Stage 1, one day post-fertilization; Stage 2, two days post-fertilization; Stage 3,
three days post-fertilization and Stage 4, four days post-fertilization. The embryos for the normal study were then prepared for light microscopy, transmission electron microscopy and scanning electron microscopy. The embryos for the LAS study were treated with LAS and then prepared for light and electron microscopy.

**Treatment with LAS**

One hundred fathead minnows were selected at each of the five stages. They were subjected to a two hour incubation period in 250ml. of a 15 ppm 11.2 LAS solution. The LAS used was 62.4% active. These embryos were then prepared for light and electron microscopy.

**Techniques for Light Microscopy**

The chorions surrounding each of the embryos were removed with forceps, immediately placed on a glass slide and cut into smaller fragments. One drop of methylene blue (0.3% solution) was placed on the fragments for staining, followed by the placement of a coverslip. Slides were observed with a compound microscope at 1000x magnification, under oil immersion. Photographs were taken immediately.

**Techniques for Transmission Electron Microscopy**

The chorions were fixed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate to pH 7.4 and post-fixed in similarly buffered 1% OsO4. After dehydration in ethanol, the specimens were embedded in Epon 812 and
sectioned with a Porter-Blum MT-1 ultramicrotome. After staining on the grid with lead citrate (Reynolds, 1963), the sections were observed with an RCA EMU-3F electron microscope at 50kV accelerating voltage. The sectioned tissue for transmission electron microscopy was oriented for both cross and tangential sections.

Techniques for Scanning Electron Microscopy

The chorions were fixed in 3% glutaraldehyde and buffered just as for transmission electron microscopy. The specimens were then dehydrated by using a graded series of ethanol concentrations and dried by the Freon critical point drying method. The specimens were placed on a rotary vacuum evaporator and coated with carbon and gold. The specimens were then viewed on a Mini-Sem scanning electron microscope. The tissue for scanning electron microscopy was oriented for inside and outside surfaces as well as cross section.

Data Collection

Photomicrographs of each of the five stages were taken, both of the normal and the LAS treated chorions. Photomicrographs were also taken of the micropyle at each of the five stages with the scanning electron microscope. The 10,000x micrographs were used to calculate distance between pores for each of the five stages, both under normal conditions and with LAS treatment. This magnification was chosen since the distance between
pores appears clearer and easier to measure. The thickness of each of the chorions of the five stages was also calculated, as well as changes in the thickness of the layers.
EXPERIMENTAL DATA

The chorion of the fathead minnow is approximately 1.0 mm in diameter (Figs. 1 and 2). The cut edge of the chorion (Fig. 3), when viewed in cross section, demonstrates a lamellar structure (Fig. 4).

I. Ultrastructure of the Normal Chorion

A) Stage F - Day of Fertilization

1) Light Microscopy

The chorion as viewed under oil immersion (Fig. 5) appears as a sieve-like membrane and the apparent pores seem to penetrate through the chorion.

2) Transmission Electron Microscopy

The cross section of the chorion of the Stage F embryo (Fig. 6) demonstrates a multilayered membrane with an average thickness of 0.010 mm. The outer surface is composed of a profile of ridges that covers an inner substructure of nineteen lamellae. These nineteen lamellae together measure 0.0097 mm. Each lamella appears to have an altering fiber orientation, similar in appearance to
the longitudinal and smooth muscle arrangement in the gastrointestinal tract. Beneath these nineteen lamellae of the inner layer is the cortical layer of the chorion. This layer is composed of a different, more electron dense, material. This cortical layer has an average thickness of 0.0003 mm. The inner surface also appears to be composed of a series of ridges, just as observed in the outer surface.

The tangential sections of the Stage F chorion (Figs. 7 and 8) evidence a fibrous meshwork in the inner layers of the chorion. Running within the meshwork are a series of microtubules (Fig. 8), within which inclusions are frequently found.

3) Scanning Electron Microscopy

The outer surface of the Stage F chorion was scanned at 2000, 4000, 7000 and 10,000x. The 2000x micrograph (Fig. 9) depicts a surface of regular indentations. The 4000x micrograph (Fig. 10) depicts the apparent pores viewed under light microscopy (Fig. 5). Under 7000x and 10,000x (Figs. 11 and 12), it can be seen that these indentations do not appear to be true perforations. These regular depressions give a honeycomb-like appearance to
the surface of the chorion.

The inner surface of the chorion of Stage F was scanned at 7000 and 10,000x, (Figs. 13 and 14). The inner surface appears to be true perforations into the cortical layer of the chorion.

B) Stage 1 - One Day Post-Fertilization

1) Light Microscopy

The chorion of the Stage 1 embryo has the same appearance as the Stage F chorion viewed under oil immersion (Fig. 15).

2) Transmission Electron Microscopy

The cross section of the chorion of the Stage 1 embryo (Fig. 15) demonstrates a multi-layered membrane with an average thickness of 0.00105 mm (10.5 microns). The outer surface also has ridges, covering an inner lamellar layer of nineteen lamellae. These nineteen lamellae together are 10.1 microns thick. There is also an inner cortical layer that is 0.4 microns thick. The Stage 1 chorion strongly resembles the Stage F chorion, only being slightly thicker in both the lamellar and cortical layers.

The tangential sections of the Stage 1 chorion (Figs. 17 and 18) show regularly
dispersed bundles that overlap and form a meshwork. The fibers appear to be oriented in a very ordered way (Fig. 18), displaying a circular arrangement.

3) Scanning Electron Microscopy

The outer surface of the Stage 1 chorion was scanned at 2000, 4000, 7000 and 10,000x. The 2000 and 4000 micrographs (Figs. 19 and 20) show a regular surface appearance, very similar to that of the Stage F chorion. At 7000 and 10,000x (Figs. 21 and 22), regular depressions are seen in the surface.

The Stage 1 chorion inside surface was also scanned at 7000 and 10,000x (Figs. 23 and 24). The "bores" in the inside surface appear to perforate into the inside of the chorion. The honeycomb effect that is seen on the outside surface is not present on the inside.

C) Stage 2 - Two Days Post-Fertilization

1) Light Microscopy

The Stage 2 chorion, viewed under oil immersion (Fig. 25), appears very similar to the Stage F and Stage 1 chorions seen with the light microscope.

2) Transmission Electron Microscopy

The thickness of the Stage 2 cross section
is approximately 11 microns (Fig. 26), showing an increase over the previous stages. The inner layer is 10 microns thick at this stage, and the cortical layer is much more distinct than in the previous stages, and appears to be separated from the other layer of the chorion.

The tangential sections of the Stage 2 chorion (Figs. 27 and 28) demonstrate the fibrous lamellae seen in the cross section. A microtubule with some type of inclusion is seen within the fibrous structure of the chorion. (Fig. 27).

3) Scanning Electron Microscopy

At 2000 and 4000x (Figs. 29 and 30), the outer surface of the Stage 2 chorion looks quite similar to the previously described stages. The micrographs taken at 7000 and 10,000x (Figs. 31 and 32) evidence an outer surface that is quite different from that seen in earlier stages. The distinct honeycomb surface that was observed at Stages F and I is no longer visible. The surface appears to be pitted with crater-like indentations.

The inside surface of the Stage 2 chorion viewed at 7000 and 10,000x (Figs. 33 and 34) shows the same "pore-like" surface as seen
in the previously described stages.

D) Stage 3 - Three Days Post-Fertilization

1) Light Microscopy

The chorion of the Stage 3 embryo, when viewed under oil immersion, (Fig. 35) gives the impression of a porous membrane, showing the same structure seen in earlier stages.

2) Transmission Electron Microscopy

The cross section of the Stage 3 chorion (Fig. 36) demonstrates a multi-layered 11 micron thick structure. The inner layer shows a thickness of 9.5 microns, and the cortical layer has increased to 1.5 microns thick.

The tangential section shows the fibrous, swirling appearance of the inner lamellae of the chorion (Figs. 37 and 38). Note the different fiber orientations seen in Figure 38.

3) Scanning Electron Microscopy

The outer surface of the Stage 3 chorion at 2000x (Fig. 39) looks quite similar to the previously described stages. At 4000x (Fig. 40), the outer surface takes on a completely different appearance. A regular appearance of indentations emerge within the elevations on the surface. The chorion appears to take on a porous appearance. At 7000 and 10,000x (Figs.
41 and 42) these "pores" are easily visible.

The inner surface of the Stage 3 chorion has the same previously described perforations (Figs. 43 and 44). There now seem to be areas on the inner surface that are devoid of perforations.

E) Stage 4 - Four Days Post-Fertilization

1) Light Microscopy

The Stage 4 chorion viewed under oil immersion (Fig. 45) has the same appearance of a porous, sieve-like membrane described for the other stages.

2) Transmission Electron Microscopy

The cross section of the Stage 4 chorion is 10.6 microns thick. The inner lamellar layer is 6.9 microns thick and the cortical layer is 3.7 microns thick (Fig. 46). This Stage 4 cross section presents quite a different picture of the chorion. The lamellar layers are still present, but the lamellae seem to become progressively thinner toward the inside. The cortical layer is shown to be much thicker than in previous stages, and makes up almost one-third of the entire thickness of the chorion.

The tangential sections of the inner layers of Stage 4 chorion (Figs. 47 and 48) show the same fibrous appearance demonstrated by the
cross section. The different fiber orientations (Fig. 47) of the successive lamellae are readily seen. It appears as if each individual fiber is separated from the others. A small microtubule (Fig. 48) is seen within the fibrous meshwork of the chorion. This is a much smaller microtubule than was seen in Figure 8 at the same magnification. There may be a series of microtubules, a type of network of small and large tubules running within the fibrous mesh of the chorion.

3) Scanning Electron Microscopy

The outer surface of the Stage 4 chorion was scanned at 2000, 4000, 7000 and 10,000x. The 2000x micrograph (Fig. 49) depicts the same regular surface of indentations seen in previous stages. The 4000x micrograph (Fig. 50) shows the regular appearance of indentations within the elevations, as seen in the Stage 3 chorion at 4000x (Fig. 40). These indentations within the elevations are more readily seen at 7000 and 10,000x (Figs. 51 and 52).

The inner surface of the Stage 4 chorion has the same perforations that were seen in all of the stages, evidenced in Figures 53, 54, at 7000 and 10,000x. The inner surface appears
much smoother than the outer surface and the perforations seem to penetrate into the chorion.

II. Ultrastructure of the LAS Treated Chorion

A) Stage F - Day of Fertilization

1) Light Microscopy

The LAS-treated chorion viewed under oil immersion has the same appearance as viewed in the four stages of the normal chorion. It looks like a sieve membrane, and the apparent pores appear to penetrate through the chorion.

2) Transmission Electron Microscopy

The cross section of the chorion of the Stage F embryo (Fig. 56) demonstrates a multilayered membrane with an average thickness of 8 microns. The inner lamellar layer is 7.6 microns wide and the inner cortical layer is 0.4 microns wide. There appear to be perforations within the lamellar layer, and the lamellae are of various thicknesses. There is a definite change from the picture of the Stage F normal chorion (Fig. 6). It seems that LAS has some altering effects on the fibrous layering of the chorion.

The tangential sections of the Stage F chorion after LAS treatment have the same appearance of a fibrous meshwork (Figs. 57 and 58),
but note that the fibers seem to be disin­
tegrating. The fibers that were sharply de­
lineated in the normal chorion now appear to
be diffuse and not as distinct.

3) Scanning Electron Microscopy

The outer surface of the Stage F chorion
treated with LAS (Figs. 59, 60, 61, and 62)
depict a surface of regular indentations, just
as viewed for this same stage in the normal
chorion. The 4000x micrograph (Fig. 60) de­
monstrates the apparent pores viewed under the
light microscopy (Fig. 55). At 7000 and 10,
000x these apparent pores give a honeycomb-
like appearance to the outer surface of the
chorion.

The inner surface (Figs. 63 and 64) shows
a surface that is much smoother than the outer
surface. The depressions appear to be true
perforations into the inner layers of the chorion.

B) Stage 1 - One Day Post-Fertilization

1) Light Microscopy

The chorion of the Stage 1 LAS-treated
chorion has the same appearance of a sieve­
like membrane (Fig. 65).

2) Transmission Electron Microscopy

The cross section of the chorion of the
Stage 1 chorion (Fig. 66) demonstrates an appearance that is quite different from the normal Stage 1 chorion. The thickness of the cross-section is only 8 microns thick, with the lamellar layer measuring 7.6 microns and the inner cortical layer is 0.4 microns. The perforations that were noted in the Stage F chorion are now more numerous. There is also varying thickness of the lamellae. There is quite a great change in the LAS-treated chorion when the cross section of the normal chorion of Stage 1 (Fig. 16) is compared with that of the LAS-treated Stage 1 chorion (Fig. 66).

The tangential sections of the LAS-treated Stage 1 chorion (Figs. 67 and 68) demonstrate a change in the fibrous arrangement of the inner layers of the chorion. The orderly arrangement of fibers seen in the normal chorion (Figs. 17 and 18) is absent. Note the different sized microtubules in Figure 67. The fibrous arrangement appears to be disrupted. (Fig. 68).

3) Scanning Electron Microscopy

The outer surface of the Stage 1 chorion treated with LAS at 2000, 4000 and 7000x (Figs. 69, 70 and 71) look very similar to the normal
chorion. At 10,000x (Fig. 72) a different surface is presented. The honeycomb appearance that was seen in the Stage 1 normal chorion is absent, and an irregular surface is seen.

The inside surface (Figs. 73 and 74) shows the same regular appearance of pores noted in the normal chorion. It appears that LAS has its major effects on the inner lamellar layers of the chorion and some effect on the outer surface in this stage.

C) Stage 2 - Two Days Post-Fertilization

1) Light Microscopy

The Stage 2 chorion, viewed under oil immersion (Fig. 75), has the same porous appearance previously described.

2) Transmission Electron Microscopy

The cross section of the LAS-treated Stage 2 chorion (Fig. 76) presents a total thickness of 8.5 microns. The inner lamellar layer and the cortical layer are hard to distinguish. The cortical layer now appears to be composed of three or four layers and has thickness of almost 1 micron. The inner lamellar layer has many perforations in the tissue and has a thickness of 7.5 microns. The differently oriented fibers seem to be put in a disarray.
The neat, orderly arrangement of lamellae that was observed in the normal chorion is absent.

The tangential sections (Figs. 77 and 78) evidence the irregularly dispersed bundles of the lamellae. Compare this arrangement with the pattern of fibers seen in Figure 28 of the normal Stage 2 chorion.

3) Scanning Electron Microscopy

The 2000x micrograph (Fig. 79) demonstrates a different surface view of the Stage 2 chorion. There appear to be large indentations in the outer surface, even at this relatively low magnification. At 4000, 7000 and 10,000x (Fig. 80, 81 and 82), there is a surface presented that differs from both the Stage F as well as the Stage 1 chorion.

The inside surface of the Stage 2 chorion, treated with LAS, (Figs. 83 and 84) show a surface that is regularly indented with pore-like structures. There are areas seen at 7000x (Fig. 83) that are devoid of these perforations.

D) Stage 3 - Three Days Post-Fertilization

1) Light Microscopy

The light micrograph (Fig. 85) shows the porous surface previously described for the other stages.
2) Transmission Electron Microscopy

The cross section of the Stage 3 LAS-treated chorion (Fig. 86) demonstrates a thickness of 8.0 microns. The inner lamellar layer has the perforations seen in earlier stages treated with LAS, and has a thickness of 7.0 microns. The inner cortical layer appears to be differentiated into three separate lamellae and has a thickness of 1.0 microns.

The tangential sections (Fig. 87 and 88) show the irregular arrangement of fibers seen in the inner layers of the Stage 3 chorion.

3) Scanning Electron Microscopy

The outer surface of the Stage 3 LAS-treated chorion was scanned at 2000, 4000, 7000, and 10,000x (Figs. 89, 90, 91, and 92). The 2000 and 4000 micrographs look like the previous stage, but at higher magnification (Figs. 91 and 92) there is the appearance of the indentation within the elevation seen in the Stage 3 normal chorion (Figs. 41 and 42). These indentations appear to be regularly spaced and appear to penetrate through the chorion.

The inner surface of the Stage 3 chorion shows an irregular surface pattern (Figs. 93 and 94). The pore-like perforations have a more
linear appearance (Fig. 93). The inner surface appears to be not as smooth as seen in the previously described stages.

E) Stage 4 - Four Days Post-Fertilization

1) Light Microscopy

The chorion of the Stage 4 LAS-treated embryo (Fig. 95) has the appearance of a sieve-like, porous membrane.

2) Transmission Electron Microscopy

The cross section of the Stage 4 chorion presents a membrane that has undergone quite a few changes (Fig. 96). The layers all appear to be interweaved and there appears to be a lot of disruption in fiber orientation. The entire thickness of the chorion is approximately 8 microns, with the inner layer having a thickness of 6.0 microns, and the cortical layer has a thickness of 2.0 microns. The well defined lamellae of the inner layer of the chorion are no longer present.

The tangential sections of the inner layers of the chorion evidence the disrupted fiber orientation seen in the cross section. The lamellae appear to be irregularly disrupted. (Figs. 97 and 98).

3) Scanning Electron Microscopy
The outer surface of the Stage 4 chorion, LAS treated, shows a regular surface with indentations (Fig. 99). At 4000x (Fig. 100) the indentations within the elevations, observed in previous stages, is seen. At 7000 and 10,000x, this surface is even more pronounced (Figs. 101 and 102).

The inner surface of the Stage 4 chorion, LAS-treated, (Figs. 103 and 104) demonstrate a surface that has the appearance of parallel wavy lines and irregularly spaced perforations. The inner surface presents a very unpatterned arrangement.

III. Ultrastructure of the Micropyle

A) Stage F - Day of Fertilization

The micropyle viewed under scanning electron microscopy (Fig. 105) appears as a large pore through which sperm may pass for fertilization. The same lamellar arrangement of fibers is seen inside the micropylar canal. At this stage, the egg has already fertilized, and the micropyle appears to be quite open.

B) Stage 1 - One Day Post-Fertilization

The micropyle at Stage 1 viewed under the scanning microscope appears smaller than that seen in Stage F. There seems to be a
drawing together of the layers of the chorion (Fig. 106). A series of ridges that are arranged around the micropyle appear to be forming, in a ray-like arrangement.

C) Two Days Post-Fertilization

The micropylar canal at Stage 2 (Fig. 107) appears to be closed more than in the previous stages. It seems that with the drawing together of fibers there is also an invagination of fibers into the chorion.

D) Stage 3 - Three Days Post-Fertilization

The micropylar canal at Stage 3 has become smaller, and the radiating arrangement that appears around the micropyle seems to be more distinct. The appearance of spokes in a wheel is analogous to the rays surrounding the micropylar canal (Fig. 108).

E) Stage 4 - Four Days Post-Fertilization

The outer surface of the micropyle at Stage 4 shows a very distinct pattern. There is a cartwheel arrangement around the micropylar canal (Figs. 109 and 110). The micropyle itself is only a small indentation on the surface. The cartwheel arrangement shows a very ordered configuration.

IV. Distance Between Pore-Like Structures
A) Normal (control) Chorion

Table 1 shows the distance between pore-like structures for the normal chorion. Graphing these figures (Fig. 111) shows that as the age increases, the distance between pores decreases. Therefore as the age increases, the number of pores increases.

B) LAS-treated Chorion

Table 2 shows the distance between pore-like structures for the LAS-treated chorion. Comparing these figures with Table 1 shows the graph on Figure 111. Just as observed for the control chorion, as age increases, the distance decreases. The graph (Fig. 111) also shows that LAS has no effect on pore inter-distance.
DISCUSSION

The chorion serves as a barrier between the embryo and the environment. The data presented in this paper indicates that the chorion is not a static barrier. It has been shown that there is morphological change in the ultrastructure of the chorion during embryogenesis. The data also suggests a change in chorionic structure due to treatment with LAS during the various stages of embryogenesis. These changes are evident on the outer and inner surfaces as well as in the cross sections.

The observed changes in the ultrastructure and thickness of the chorion may also have an effect on the movement of materials into and out of the chorion. Biological membranes act as incomplete barriers between two separate compartments. They are incomplete in that a transfer of molecules across them and between them may still occur. This transfer has been postulated to be of two types, diffusion and carrier transport (Neame et al, 1972).

Diffusion is characterized by the net flow of molecules from a region of higher concentration to a region of lower concentration. This net flow of molecules is
simple, or passive diffusion. This diffusion process maintains a rate which is directly proportional to the solute concentration and which is directed merely by physical forces. Passive diffusion is an exergonic process and other factors affecting its rate include the molecular weights of the interacting molecules, the distance the molecules must travel and the temperature of the entire molecular system itself (Guyton, 1971). Since there is a change in the thickness of the chorion during embryogenesis, this change would reflect a change in the rate of passive diffusion.

Diffusion through membrane barriers is a common entity in all living systems, however, diffusion alone cannot explain all molecular transfer through these barriers. Therefore, a second type of transfer mechanism, carrier transport, has been postulated. The movement of molecules through a barrier at a rate which is greater than possible by diffusion alone and which displays enzyme kinetic properties is called carrier transport.

The general carrier transport mechanism entails the attachment of solute molecules to carrier sites thereby forming complexes which move across the membrane. The solute molecules may then be released on the other side of the membrane. The carrier has been postulated as being either a structure in the membrane or a conformational change in the membrane. A change in the ultra-
structure of the chorion may result in a change in the number or mobilization of active sites on the membrane. This would have an effect on the active transport of materials through the chorion.

Detergent surfactants are capable of adsorbing to cell membranes which may then lead to the depolarization of the cell membrane. In this situation, the net surface change of the membrane could be changed (Kishimoto and Adelman, 1964). It has been noted that there is an increase in the adsorption of different materials from the colon of mammals after oral administration of surfactants (Lish and Weikel, 1959). Since surfactants have this effect on membranes, it is conceivable that LAS may change the chorionic membrane, chemically or morphologically, thus decreasing the rates of diffusion or carrier transport through the membrane. It is possible that LAS has an effect of immobilizing the carrier sites in the membrane, since LAS did show an effect on the structure of the chorion. The disruption of layers could cause a change in the number of available active sites.

Manner and Thompson (1974) suggested that the differential response at different stages of embryogenesis to comparable dosages of LAS can be explained as a differential susceptibility of embryonic tissue to this pollutant. Although this may be true, this paper
suggests that the changing structure of the chorion observed during embryogenesis may allow different amounts of pollutants to pass into the embryo at different stages. This would account for the differential response at the various stages.

Manner and Muehleman (1975) showed that both diffusion and uptake of tritiated uridine increase during fathead minnow embryogenesis. They suggested a change in the permeability of the chorion surrounding the embryo during the developmental period. They also showed that the effect of LAS on the diffusion and uptake of tritiated uridine is one of inhibition, since in all developmental stages LAS decreased the amount of tritiated uridine which enters through and remains within the surrounding chorion. This paper suggests that definite changes in the structure of the chorion could account for the changes in permeability during embryogenesis and with the treatment of LAS. I suggest that this altered permeability arises from the change in diffusion and carrier transport caused by ultrastructural changes in the chorion.

Muehleman (personal communication) studied the number of pore-like structures visible under the compound microscope. She showed an increase in the number of pores during embryogenesis and that LAS has no effect on the number of pores. This study indicates that the distance between pores decreases during embryogenesis, and LAS has
no effect on this pore distance; therefore, this is in agreement with the work of Muehleman. LAS appears to have its greatest effect on the inner layers of the chorion.

Hisaoka (1958) described the chorion as a "leakage membrane" with pores that penetrate through the chorion. His study was only concerned with light microscopy. This paper has shown that under light microscopy, the chorion does appear somewhat porous. Using electron microscopy, it has been shown that these pores do not penetrate through the chorion.

Yamamoto and Yamagami (1975) showed the morphological changes in the chorion brought about by the hatching enzyme. They suggested that no significant changes in the morphological features, especially in the lamellar structure, occurred until the release of the hatching enzyme. They showed that the hatching enzyme caused a digestion of the inner layer of the chorion. They suggested that the hatching enzyme attacks some restricted peptide bonds in the framework proteins of the inner layer of the chorion. The result of the hatching enzyme or some other enzyme may be the cause of the thickened cortical layer during embryogenesis. I suggest that the thickening of the cortical layer is the result of some type of enzymatic disruption of the lamellar structure of the chorion, leaving the partially dissolved framework of the lamellae. This framework of partially dis-
solved skeleton produces the different, more electron
dense layer that is seen to increase in thickness during
embryogenesis.

This study suggests that the micropyle must also
be considered when studying the permeability of the chorion
during embryogenesis. The mechanism of how the micro­
pylar canal closes is still elusive. Future research
should try and determine the ultrastructure of the chorion
and the micropyle before fertilization. It would be in­
teresting to compare the chorion of the fathead minnow
with that of other teleost. At present, there is no way
to identify egg types of different species. The ultra­
structure of the chorion may provide a method for ident­
ifying and classifying different egg types.
PLATE 1

Figure 1 - Outer surface of the chorion (S.E.M.) X 50

Figure 2 - Outer surface of the chorion (S.E.M.) X 100
PLATE 2

Figure 3 - Outer surface of the chorion (S.E.M.) X 400

Figure 4 - Cross section of the chorion (S.E.M.) X 1200
PLATE 3

Figure 5 - Outer surface of the Stage F chorion (light microscopy) X 4000

Figure 6 - Cross section of the Stage F chorion (T.E.M.) X 10,000
PLATE 4

Figure 7 - Tangential section of the inner layers of the Stage F chorion (T.E.M.) X 20,000

Figure 8 - Tangential section of the inner layers of the Stage F chorion showing a microtubule with inclusions (T.E.M.) X 20,000
PLATE 5

Figure 9 - Outer surface of the Stage F chorion (S.E.M.)  
X 2000

Figure 10 - Outer surface of the Stage F chorion (S.E.M.)  
X 4000
PLATE 6

Figure 11 - Outer surface of the Stage F chorion (S.E.M.)
X 7000

Figure 12 - Outer surface of the Stage F chorion (S.E.M.)
X 10,000
PLATE 7

Figure 13 - Inner surface of the Stage F chorion (S.E.M.)
X 7000

Figure 14 - Inner surface of the Stage F chorion (S.E.M.)
X 10,000
PLATE 8

Figure 15 - Outer surface of the Stage 1 chorion (light microscopy) X 4000

Figure 16 - Cross section of the Stage 1 chorion (T.E.M.) X 10,000
Figure 17 - Tangential section of the inner layers of the chorion, Stage 1, (T.E.M.) X 10,000

Figure 18 - Tangential section of the inner layers of the Stage 1 chorion (T.E.M.) X 12,000
PLATE 10

Figure 19 - Outer surface of the Stage 1 chorion (S.E.M.)
X 2000

Figure 20 - Outer surface of the Stage 1 chorion (S.E.M.)
X 4000
PLATE 11

Figure 21 - Outer surface of the Stage 1 chorion (S.E.M.)
   X 7000

Figure 22 - Outer surface of the Stage 1 chorion (S.E.M.)
   X 10,000
Figure 23 - Inner surface of the Stage 1 chorion (S.E.M.)
X 7000

Figure 24 - Inner surface of the Stage 1 chorion (S.E.M.)
X 10,000
PLATE 13

Figure 25 - Outer surface of the Stage 2 chorion (light microscopy) x 4000

Figure 26 - Cross section of the Stage 2 chorion (T.E.M.) x 10,000
Figure 27 - Tangential section of the inner layers of the Stage 2 chorion showing a microtubule with inclusions (T.E.M.) X 20,000

Figure 28 - Tangential section of the inner layers of the Stage 2 chorion (T.E.M.) X 10,000
PLATE 15

Figure 29 - Outer surface of the Stage 2 chorion (S.E.M.)
X 2000

Figure 30 - Outer surface of the Stage 2 chorion (S.E.M.)
X 4000
PLATE 16

Figure 31 - Outer surface of the Stage 2 chorion (S.E.M.)
X 7000

Figure 32 - Outer surface of the Stage 2 chorion (S.E.M.)
X 10,000
PLATE 17

Figure 33 - Inner surface of the Stage 2 chorion (S.E.M.)
X 7000

Figure 34 - Inner surface of the Stage 2 chorion (S.E.M.)
X 10,000
PLATE 18

Figure 35 - Outer surface of the Stage 3 chorion (light microscopy) X 4000

Figure 36 - Cross section of the Stage 3 chorion (T.E.M.)
X 10,000
PLATE 19

Figure 37 - Tangential section of the inner layers of the Stage 3 chorion (T.E.M.) X 14,000

Figure 38 - Tangential section of the inner layers of the Stage 3 chorion (T.E.M.) X 12,000
PLATE 20

Figure 39 - Outer surface of the Stage 3 chorion (S.E.M.)
X 2000

Figure 40 - Outer surface of the Stage 3 chorion (S.E.M.)
X 4000
Figure 41 - Outer surface of the Stage 3 chorion (S.E.M.)
X 7000

Figure 42 - Outer surface of the Stage 3 chorion (S.E.M.)
X 10,000
Figure 43 - Inner surface of the Stage 3 chorion (S.E.M.)
X 7000

Figure 44 - Inner surface of the Stage 3 chorion (S.E.M.)
X 10,000
PLATE 23

Figure 45 - Outer surface of the Stage 4 chorion (light microscopy) X 4000

Figure 46 - Cross section of the Stage 4 chorion (T.E.M.) X 10,000
Figure 47 - Tangential section of the inner layers of the Stage 4 chorion showing the different fiber orientations (T.E.M.) X 20,000

Figure 48 - Tangential section of the inner layers of the Stage 4 chorion showing a small microtubule (T.E.M.) X 20,000
PLATE 25

Figure 49 - Outer surface of the Stage 4 chorion (S.E.M.)
X 2000

Figure 50 - Outer surface of the Stage 4 chorion (S.E.M.)
X 4000
PLATE 26

Figure 51 - Outer surface of the Stage 4 chorion (S.E.M.)
X 7000

Figure 52 - Outer surface of the Stage 4 chorion (S.E.M.)
X 10,000
PLATE 27

Figure 53 - Inner surface of the Stage 4 chorion (S.E.M.)
X 7000

Figure 54 - Inner surface of the Stage 4 chorion (S.E.M.)
X 10,000
PLATE 28

Figure 55 - Outer surface of the Stage F LAS treated chorion (light microscopy) X 4000

Figure 56 - Cross section of the Stage F LAS treated chorion (T.E.M.) X 10,000
PLATE 29

Figure 57 - Tangential section of the inner layers of the Stage F LAS treated chorion (T.E.M.) X 12,000

Figure 58 - Tangential section of the inner layers of the Stage F LAS treated chorion (T.E.M.) X 20,000
PLATE 30

Figure 59 - Outer surface of the Stage F LAS treated chorion (S.E.M.) X 2000

Figure 60 - Outer surface of the Stage F LAS treated chorion (S.E.M.) X 4000
PLATE 31

Figure 61 - Outer surface of the Stage F LAS treated chorion (S.E.M.) X 7000

Figure 62 - Outer surface of the Stage F LAS treated chorion (S.E.M.) X 10,000
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Figure 64 - Inner surface of the Stage F LAS treated chorion (S.E.M.) X 10,000
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Figure 65 - Outer surface of the Stage 1 LAS treated chorion (light microscopy) X 4000

Figure 66 - Cross section of the Stage 1 LAS treated chorion (T.E.M.) X 10,000
Figure 67 - Tangential section of the inner layers of the Stage 1 LAS treated chorion showing different sized microtubules (T.E.M.) X 12,000

Figure 68 - Tangential section of the inner layers of the Stage 1 LAS treated chorion (T.E.M.) X 10,000
PLATE 35

Figure 69 - Outer surface of the Stage 1 LAS treated chorion (S.E.M.) X 2000

Figure 70 - Outer surface of the Stage 1 LAS treated chorion (S.E.M.) X 4000
PLATE 36

Figure 71 - Outer surface of the Stage 1 LAS treated chorion (S.E.M.) X 7000

Figure 72 - Outer surface of the Stage 1 LAS treated chorion (S.E.M.) X 10,000
PLATE 37

Figure 73 - Inner surface of the Stage 1 LAS treated chorion (S.E.M.) X 7000

Figure 74 - Inner surface of the Stage 1 LAS treated chorion (S.E.M.) X 10,000
Figure 75 - Outer surface of the Stage 2 LAS treated chorion (light microscopy) X 4000

Figure 76 - Cross section of the Stage 2 LAS treated chorion (T.E.M.) X 10,000
Figure 77 - Tangential section of the inner layers of the Stage 2 LAS treated chorion (T.E.M.) X 10,000

Figure 78 - Tangential section of the inner layers of the Stage 2 LAS treated chorion (T.E.M.) X 12,000
PLATE 40

Figure 79 - Outer surface of the Stage 2 LAS treated chorion (S.E.M.) X 2000

Figure 80 - Outer surface of the Stage 2 LAS treated chorion (S.E.M.) X 4000
PLATE 41

Figure 81 - Outer surface of the Stage 2 LAS treated chorion (S.E.M.) X 7000

Figure 82 - Outer surface of the Stage 2 LAS treated chorion (S.E.M.) X 10,000
Figure 83 - Inner surface of the Stage 2 LAS treated chorion (S.E.M.) X 7000

Figure 84 - Inner surface of the Stage 2 LAS treated chorion (S.E.M.) X 10,000
Figure 85 - Outer surface of the Stage 3 LAS treated chorion (light microscopy) X 4000

Figure 86 - Cross section of the Stage 3 LAS treated chorion (T.E.M.) X 10,000
Figure 87 - Tangential section of the inner layers of the Stage 3 LAS treated chorion (T.E.M.) X 12,000

Figure 88 - Tangential section of the inner layers of the Stage 3 LAS treated chorion (T.E.M.) X 10,000
Figure 89 - Outer surface of the Stage 3 LAS treated chorion (S.E.M.) X 2000

Figure 90 - Outer surface of the Stage 3 LAS treated chorion (S.E.M.) X 4000
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Figure 91 - Outer surface of the Stage 3 LAS treated chorion (S.E.M.) X 7000

Figure 92 - Outer surface of the Stage 3 LAS treated chorion (S.E.M.) X 10,000
Figure 93 - Inner surface of the Stage 3 LAS treated chorion showing an irregular surface pattern (S.E.M.) X 7000

Figure 94 - Inner surface of the Stage 3 LAS treated chorion (S.E.M.) X 10,000
PLATE 48

Figure 95 - Outer surface of the Stage 4 LAS treated chorion (light microscopy) X 4000

Figure 96 - Cross section of the Stage 4 LAS treated chorion (T.E.M.) X 10,000
PLATE 49

Figure 97 - Tangential section of the inner layers of the Stage 4 LAS treated chorion (T.E.M.) X 12,000

Figure 98 - Tangential section of the inner layers of the Stage 4 LAS treated chorion (T.E.M.) X 13,000
PLATE 50

Figure 99 - Outer surface of the Stage 4 LAS treated chorion (S.E.M.) X 2000

Figure 100 - Outer surface of the Stage 4 LAS treated chorion (S.E.M.) X 4000
PLATE 51

Figure 101 - Outer surface of the Stage 4 LAS treated chorion (S.E.M.) X 7000

Figure 102 - Outer surface of the Stage 4 LAS treated chorion (S.E.M.) X 10,000
Figure 103 - Inner surface of the Stage 4 LAS treated chorion showing a very irregular surface (S.E.M.) X 7000

Figure 104 - Inner surface of the Stage 4 LAS treated chorion (S.E.M.) X 10,000
PLATE 53

Figure 105 - Outer surface of the micropyle of a Stage F chorion (S.E.M.) X 3000

Figure 106 - Outer surface of a micropyle of a Stage 1 chorion (S.E.M.) X 1000
PLATE 54

Figure 107 - Outer surface of the micropyle of a Stage 2 chorion (S.E.M.) X 1000

Figure 108 - Outer surface of the micropyle of a Stage 3 chorion (S.E.M.) X 1000
Figure 109 - Outer surface of the micropyle of a Stage 4 chorion (S.E.M.) X 400

Figure 110 - Outer surface of the micropyle of a Stage 4 chorion showing a distinct cartwheel appearance (S.E.M.) X 1000
EXPLANATION OF TABLE 1

Table 1 Mean distance between centers of pore-like structures of fathead minnow embryos of five different stages during embryogenesis.
<table>
<thead>
<tr>
<th>Stage of the Embryo</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Distance between pore-like structures/mm x 10^-4</td>
<td>14.67</td>
<td>14.39</td>
<td>14.28</td>
<td>13.74</td>
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<td>15.30</td>
<td>14.45</td>
<td>14.23</td>
<td>13.45</td>
<td>12.68</td>
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<td>Mean Dist. mm/10^-4</td>
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<td>14.42</td>
<td>14.26</td>
<td>13.60</td>
<td>12.68</td>
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<td>.02</td>
<td>.02</td>
<td>.10</td>
<td>.14</td>
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Table 1
EXPLANATION OF TABLE 2

Table 2  Mean distance between centers of pore-like structures of LAS treated fathead minnow embryos of five different stages during embryogenesis.
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<th>2</th>
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<tr>
<td>Distance between</td>
<td>14.52</td>
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<td>14.15</td>
<td>13.62</td>
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<td>mm x $10^{-4}$</td>
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Table 2
EXPLANATION OF FIGURE 111

Figure 111 Comparison of the mean distance between pore-like structures of control and LAS treated fathead minnow chorions of five different stages during embryogenesis.
LITERATURE CITED


Hisaoka, K.K. 1960. Further studies on the embryonic


of copper to the fathead minnow (Pimephales promelas) in soft water. J. Fish. Res. Bd., Canada, 26: 2449-2457.


The thesis submitted by Mark Xavier VanCura has been read and approved by the following committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given full approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

May 3, 1976

Date

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