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STUDY OF THE DEVELOPMENTAL STAGES OF BLUEGILL (*LEPOMIS MACROCHIRUS*) EGGS USING SELECTED HISTOLOGICAL TECHNIQUES

By

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ABSTRACT

STUDY OF THE DEVELOPMENTAL STAGES OF BLUEGILL (LEPOMIS MACROCHIRUS) EGGS USING SELECTED HISTOLOGICAL TECHNIQUES

By

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Bluegill are important gamefish for stocking farm ponds and lakes; but, their high reproduction rates can cause stunting. Polyploid induction may be used for population control. The objectives of this study were: 1) to develop histological techniques to monitor egg development to determine the critical time for the production of polyploid bluegill and 2) to observe differences between the development of normal eggs and eggs that received polyploid induction treatments.

Standard histological techniques and laser scanning confocal microscopy (LSCM) were not effective in monitoring chromosomal changes in fertilized eggs. However, LSCM could be used to observe developmental changes in the eggs. Blastodiscs were not observed to divide in eggs that received induction treatments during the first 60 minutes postfertilization. Polyploid induction treatments may have produced a shock to the cell that required a recuperation period, cell division was inhibited in polyploid eggs, or treatments had a lethal effect on the eggs.

This thesis is dedicated to my loving husband Bryan R. Brunner and to my little boy Bryan José for all the reasons that only they know.

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KEY TO SCIENTIFIC NOMENCLATURE

Common name	Scientific name
Atlantic salmon	*Salmo salar*
Bluegill	*Lepomis macrochirus*
Blue tilapia	*Oreochromis aureus*
Brook trout	*Salvelinus fontinalis*
California roach	*Hesperoleucus symmetricus*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tshawytscha*
Common carp	*Cyprinus carpio*
Fathead minnow	*Pimephales promelas*
Green sunfish	*Lepomis cyanellus*
Largemouth bass	*Micropterus salmoides*
Nile tilapia	*Oreochromis niloticus*
Rainbow trout	*Oncorhynchus mykiss*

INTRODUCTION

The Centrarchid family is indigenous only to North America and consists of 30 fish species. Several species have been studied extensively by fishery biologists and their systematics have been carefully considered (Roberts, 1964). However the family has received only scant attention from cytologists (Roberts, 1964). The bluegill (Lepomis macrochirus, Rafinesque) belongs to this family and is the largest lepomine sunfish found in Michigan (Janssen, 1974). It has a wide range of distribution and is found in lakes and streams from western New York throughout the Great Lakes region and Mississippi Valley to northern Mexico, and along the Atlantic Coast all the way from New York to Florida (Morgan, 1951). In Michigan it is found primarily in lakes and reservoirs and is less abundant in the larger rivers.

Bluegill are the favorite panfish of North American anglers and are the most frequently pursued gamefish in the United States (Janssen, 1974). Because it easily adapts to pond conditions, it is also one of the favorite fishes for stocking farm ponds (Morgan, 1951). They may grow to 4 inches and reproduce in 1 year, and some may spawn more than once during the summer (Morgan, 1951).

The high fecundity of bluegill is an important factor in its selection as a forage fish for largemouth bass in lakes and ponds (Morgan, 1951). In spite of their desirable traits, their early sexual maturity and high rates of reproduction can cause overpopulation problems and negatively influence growth rate (Childers, 1967; Murnyak et al. 1984). Once sexual maturity is reached, fish must expend metabolic energy between somatic and reproductive growth; this results in a reduction in growth rate (Murnyak et al. 1984).

Frequently bluegills dominate the biomass of the lakes and ponds they inhabit. Where they predominate numerically, they are prone to stunting. Murnyak et al. (1984) defined stunting as an exhibition of an individual growth rate below the potential for the species. Some of the factors that can cause stunting are high reproductive rates, low predation rates and limited supply of food items. In the case of bluegills in overcrowded situations, the cessation of growth occurs when 3 to 5 inches in length is attained. Although the average length for males is 5.76 inches and for females 6.62 inches, adult bluegill have the potential to grow to lengths over 10 inches and a weight of over a pound (Morgan, 1951). The world record hook-and-line bluegill was 4 lb. 12 oz. caught at Ketona Lake, Alabama on April 9, 1950 by T.S. Hudson (IGFA, 1990).

Overpopulation and subsequent stunting can be a problem in both aquaculture and natural fish communities (Lagler and Steinmetz, 1957; Childers, 1967; Wohlfarth and Hulata, 1983; Murnyak et al. 1984). Stunting affects fisheries by reducing the number of acceptable sized bluegill. Strategies that have been developed to overcome this problem include nongenetic and genetic techniques (Wohlfarth and Hulata, 1983). Seining, trapping and partial or complete poisoning of bluegill populations and nests are some of the nongenetic methods that have been used to reduce bluegill numbers; but, these techniques are labor intensive and costly. Chromosomal manipulation by polyploid induction is one of the most recent genetic techniques used as a means of population control in fishes (Bidwell et al. 1985; Myers, 1986; Thompson et al. 1987; Don and Avtalion, 1988; Ihssen et al. 1990).

Temperature (cold and heat), chemicals and hydrostatic pressure shocks have been used to induce polyploidy in fishes (Ihssen et al. 1990). In order for these methods to be successful, an understanding of the pre- and postfertilization stages of development of the egg is necessary. Little work has been done in this area with the bluegill.

Interest in reproductive control by polyploid induction arises from the fact that triploidy in fish causes sterility. It has been argued that sterility has a positive

impact on food conversion and growth rate because the energy normally used for gametogenesis in a fertile fish may be used in triploids to increase the quantity of edible tissue (Don and Avtalion, 1986).

Triploidy provides functional sterility because the normal pairing of chromosomes during meiosis I is impeded by the presence of three homologous chromosomes. This results in the uneven or aborted separation of chromosome triplets which inhibits the early development of gametes (Myers, 1986).

Purdom (1983) found that the sterility effect of triploidy was manifested in different ways depending on the fish species. Some species of fish may exhibit retarded and reduced gonad development and the production of nonviable gametes, other species may exhibit degenerated gonads and gametes and some may exhibit sexual characteristics and normal gonads but produce nonviable gametes. In the cases where triploidy causes gonad development to be reduced, an increase in somatic growth has been reported (Myers, 1986). Examples of gonad reduction in size and function are found in triploid Atlantic salmon, rainbow trout and Coho salmon (Benfey and Sutterlin, 1982).

Conflicting reports of triploid growth potential are found throughout the literature. In animals, the nuclear and cell sizes increase in proportion to the increase in chromosome number, so it is expected that triploids should

show an increased size over normal diploids (Gold, 1979). Some researchers state that triploids grow faster and attain greater weights than diploids (Valenti, 1975) while others argue that differences in growth rate can only be seen after sexual maturity (Gervai et al. 1980; Don and Avtalion, 1986). However, for some fish species it has been observed that the increased cell size of triploid individuals is compensated by a reduction in the number of cells per organ; therefore, they are not bigger than the normal diploid individuals (Gold, 1979).

Two methods for inducing triploidy have been suggested: 1) inhibition of the second meiotic division in the fertilized eggs and 2) crossing a tetraploid with a diploid organism (Myers, 1986). Both of these techniques have been used to produce triploid fishes with varying levels of practicality and success (Ihssen et al. 1990).

The inhibition of the second meiotic division will result in eggs with two sets of maternal chromosomes. Fertilization by haploid sperm then produces triploid individuals. In fishes, the second meiotic division is completed shortly after fertilization (Ginzburg, 1972). This division can be prevented by using a treatment that causes depolymerization of microtubules which form the spindle apparatus (Purdom, 1983). The spindle apparatus is responsible for separation of chromosomes during cell division.

The use of tetraploids as a method of producing triploids in fish has been suggested as a way of avoiding conventional induction techniques (Chourrout et al. 1986; Chourrout and Nakayana, 1987). It has been argued that conventional triploid induction techniques may cause abnormal embryonic development (Myers, 1985). Tetraploidy has been achieved by shocking the fertilized eggs before metaphase of the first mitotic division (Myers, 1986). The success of tetraploid induction for each species depends on the timing (before mitotic metaphase) of the treatment, intensity of the treatment and the duration of exposure (Myers, 1986).

The objectives of this study were:

1) To develop histological techniques to monitor egg developmental stages in order to determine the critical stage for production of triploid and tetraploid bluegill.
2) To observe and describe differences, if any, between the development of normal eggs and the development of eggs that received pressure or cold shock treatment for the production of triploid and tetraploid bluegill.

LITERATURE REVIEW

Reproduction control is desirable to channel available energy to promote efficient growth in fishes. Growth is controlled to some extent by environmental conditions such as availability of food and space. Limitation of these factors can result in slow growth and stunting (Murnyak et al. 1984). There is an inverse relationship between fish density and growth rate (Smith, 1980). Density affects growth through food and space shortages. Growth is density-dependent with greater growth at lower fish density (Murnyak et al. 1984).

Based on scale analysis, Murnyak et al. (1984) found that growth rates of non-stunted and stunted bluegill were similar before stunting. They also found that under improved environmental conditions non-stunted bluegill grew significantly better than stunted bluegill. They argued that stunting could have prolonged detrimental effects on bluegill by reducing their capacity for growth. Stunted bluegill grew more slowly and did not realize their full growth and yield potential.

Control of Reproduction in Fishes

Research efforts to improve fish growth rate have concentrated on different methods of reproduction control. Techniques for controlling reproduction may be classified as genetic and nongenetic.

Some of the nongenetic techniques to control bluegill reproduction have been seining, trapping and poisoning of populations and nests. These methods are labor intensive and costly. Other methods that have been used with bluegill and other species, like tilapia, include the use of predators, monosex culture, reproductive sterilization, cage culture and high density stocking rates (Table 1).

The use of predators to control bluegill reproduction has been shown to be inadequate or too effective under different ecological situations and stocking densities (Huet, 1970). For example, in very shallow and weedy waters, the use of largemouth bass as a predator may be inadequate because the bluegill can make themselves inaccessible by hiding in the weeds. Conversely, if predators are stocked with bluegill in water where there is little or no cover, predators can cause a "negative" effect on the prey population. The use of predators in this case would be considered too effective (Huet, 1970).

Monosex culture has been used with tilapia in an effort to control overpopulation. This type of culture can be

Table 1. Techniques used to control overpopulation in aquaculture and natural fish communities.

Technique	Author
Nongenetic	
Seining, trapping and poisoning of populations and nests	Childers, 1967 Murnyak et al. 1984
Predators	Huet, 1970
Monosex culture	Pandian and Varadaraj, 1987
Reproductive sterilization	Nelson et al. 1976
Cage culture	Rifai, 1980
Genetic	
Hybrids	Childers, 1967 Dawley, 1987
Sex-reversal	Pandian and Varadaraj, 1987
Chromosomal manipulation by polyploid induction	Thorgaard et al. 1981 Purdom, 1983 Myers, 1986

achieved by sexing the fingerlings and stocking one sex, or by eliminating one sex from the population by sex reversal or hybridization (genetic methods). Sexing tilapia fingerlings can be accomplished by examination of the urogenital papillae; two orifices are present in the female and one in the male. The major disadvantage of this method has been human error. One female introduced into a pond can undo all the labor involved in sexing the fish (Pandian and Varadaraj, 1987). Also, not all juvenile fish exhibit sexual dimorphism. This is true for bluegill and many other fishes (Morgan, 1951).

Reproductive sterilization by irradiation has not yielded practical results (Nelson et al. 1976). Examination of the gonads from irradiated fish indicated neither obvious depletion of the germ cells nor evidence of necrobiosis even when the irradiation levels were greater than the single lethal dose for the fish (Nelson et al. 1976). This indicated that germ cells are very resistant to radiation.

Reproduction can be controlled in culture ponds by cage culture. However, cage culture appears to be of limited commercial value due to the additional cost of the materials needed to build the cages and the amount of cages necessary to raise enough fish to make a profit (Rifai, 1980). Controlling reproduction by high density stocking rates has the disadvantage of producing small fish size at harvest time.

Genetic methods to control reproduction include interspecific hybridization, the use of sex reversed individuals as brood stock and chromosomal manipulation (Table 1). The only genetic method for reproductive control attempted with sunfish has been the production of hybrids (Childers 1967; Dawley, 1987).

Some interspecific and intergeneric hybrids that result in nearly all male progeny have been reported for bluegill (Childers, 1967). Although hybridization techniques to produce nearly all male fish have been developed during the last 20 years, their applicability to monosex culture has remained limited due to: 1) the difficulty in maintaining pure parental stocks that consistently produce 100% male offspring, 2) poor spawning success (Childers, 1967) and 3) incompatibility of breeders resulting in low fertility (Childers, 1967).

Prior to the discovery that some sunfish hybrids were fertile, their use was proposed as a way to increase fish size and control reproduction. Hubbs and Hubbs (1933) concluded that sunfish hybrids were sterile. In more recent studies, it has been found that the majority of the sunfish crosses are fertile (Krumholz, 1950; Lagler and Steinmetz, 1957; Dawley, 1987) and that in some cases the total length of the hybrids was not significantly different from their parents (Childers, 1967).

The use of sunfish hybrids has sometimes resulted in the same difficulties as using the parental species because their reproductive capacity and their ability to survive allows them to become so abundant that they are unable to grow to sizes large enough to be of value for fishermen (Childers, 1967). However, not all hybrids show high reproductive capabilities. Dawley (1987) reported that some hybrids show reduced fertility and Childers (1967) reported that for some hybrids the sex ratio is almost 100% males. Childers (1967) argued that some F1 hybrids appear to be highly vulnerable to capture by hook-and-line and that large populations of hybrids can be almost completely eliminated in a few days when subjected to moderate fishing pressure. Childers (1967) also found that certain kinds of F1 hybrids appeared to be unable to produce sizeable F2 populations in ponds containing largemouth bass; but, this can vary with vegetation abundance.

Hormone treatment for producing sex-reversed individuals has been widely use with tilapia (Pandian and Varadaraj, 1987). This technique interferes with the sex-determining mechanism in half of the population. For example, if a population of only males is desired, an androgen is administered through diet or water to a normal population of young fish. The genetic females are induced to develop as functional males, while genetic males are not affected. Because gonads are irreversibly differentiated

during a specific period in the larval development, the sex determination must be manipulated during the post-hatching differentiation period (Nelson et al, 1976). The desired hormonal action depends on the efficacy and dose of the steroid, method of administration, and time and duration of the treatment.

Some researchers have failed to obtain 100% sex reversed progeny using hormone treatments primarily for two reasons: 1) failure to synchronize the hormone treatment duration with that of gonadal differentiation and/or 2) failure in the feeding regime. Fry in the last peck of hierarchal order fail to receive the effective minimum hormone treated diet to ensure complete sex reversal. In these individuals, the differentiation process results in the production of intersex or even female fish. Stunted growth and liver malformation have also been observed in sex reversed fish subjected to high doses of androgens (Pandian and Varadaraj, 1987).

Use of Polyploidy for Control of Reproduction in Fishes

The use of induced polyploidy as a genetic technique for reproductive control has attracted considerable attention in recent years (for review see Ihssen et al. 1990). The majority of studies have dealt with the induction of triploidy in a wide variety of fish. Producing tetraploid fish is fairly recent and remains a desirable

goal because of its potential for further production of triploids (Thorgaard et al. 1981; Purdom, 1983; Myers, 1986). Several techniques have been used to induce polyploidy in fishes. These treatments have included temperature (cold and heat), chemicals and hydrostatic pressure shocks (Ihssen et al. 1990).

Polyploid organisms possess complete chromosome sets in excess of the two sets normally found in diploids. Triploids have showed enhanced growth due to their sterility and altered cell physiology (Valenti, 1975). Two methods have been used to induce triploidy: 1) inhibition of the second meiotic division in the fertilized eggs and 2) crossing a tetraploid and a diploid organism (Myers, 1986).

For the induction of triploidy, temperature shock has been the most commonly used method due to its simplicity. This treatment causes the inhibition of the second meiotic division in the fertilized egg. In salmonids, a heat shock of 27-32°C applied 10 to 20 min postfertilization produced a satisfactory survival and high induction efficiency (Chourrout, 1980). Heat shock has been successful with the common carp (Gervai et al. 1980) and with Oreochromis sp. (Valenti, 1975). Wolters et al. (1982) obtained 100% induction of triploidy in catfish with the use of cold shock. Valenti (1975) found cold shocks of 11°C (75% triploid) were superior to heat shocks at 38°C (10% triploid) in O. aureus. However, Chourrout and Itskovich

(1983) obtained 95% triploid in O. niloticus using a heat shock of 40.5°C.

Hydrostatic pressure has been used to induce triploidy primarily in the salmonids. Allen and Myers (1985) found that pressure treatments of 8000 psi for 10 min in Chinook salmon resulted in more consistent triploid induction than with a heat shock of 29°C for 15 min. But Benfey and Sutterlin (1982) found heat shocking Atlantic salmon eggs was more effective than pressure treatment in inducing triploidy.

It has been argued that the treatment for triploid induction (temperature, pressure or chemical shock) may cause abnormal embryonic development for which the organism is unable to compensate (Myers, 1985). Crossing tetraploids with diploids as a method of producing triploids in fish has been suggested as a way of avoiding the negative side effects of conventional induction techniques. It has been reported that the growth of triploid fish produced by crossing tetraploids with diploids was superior to triploids produced via inhibition of the second meiotic division in the fertilized egg (Myers, 1986).

Tetraploidy has been achieved via suppression of the first postfertilization mitotic division. The use of temperature shocks, cytochalasin B and hydrostatic pressure to inhibit first cleavage have been attempted in a variety of fishes (Ihssen et al. 1990) and amphibians (Fischberg,

1959). Fischberg (1959) was able to achieve 93.7% tetraploidy in newts by applying a 36 to 37°C shock for 10 min during the formation of the cleavage furrow. Thorgaard et al. (1981) produced tetraploid rainbow trout by shocking eggs at 36°C for 10 min at 5 to 6 hours postfertilization. Tetraploidy can also be induced in rainbow trout by applying a less intense shock, 28°C, for 14 min at the time of first cleavage (Chourrout, 1982).

Cytochalasin B acts primarily in preventing the formation of actin fibers in the cleavage furrow but also affects respiration, glucose uptake and protein synthesis (Grant, 1978). Refstie et al. (1977) and Allen and Stanley (1979) produced mosaic salmonids after cytochalasin B treatments. The mosaics were comprised of haploid, diploid, triploid and tetraploid cells. The production of mosaics could have been the result of lack of synchronization between the stage of development of the egg and the application of the treatment.

Hydrostatic pressure has proved to be an effective tetraploid induction method when the shock has been applied during the metaphase-anaphase period prior the cleavage. Chourrout (1984) used a hydrostatic pressure shock treatment of 7000 psi for 4 min to achieved 100% tetraploid induction in rainbow trout. At one year of age some tetraploid males reached maturity and were mated with diploid females.

Triploids produced this way were larger than those produced via meiotic inhibition (Chourrout, 1984).

Naturally Occurring Polyploid in Fishes

The increased interest in the study of fish chromosome and cytology may be caused by the new awareness of the benefits derived from genetic manipulation in fish. As previously mentioned, chromosomal manipulation by polyploid induction is one of the most recent genetic techniques used as a means of population control in fishes. Natural polyploidism is extremely rare among bisexual vertebrates but rather common in plants (Gold, 1979). Muller (1925) stated that one reason for this is that animals usually have two sexes which are differentiated by means of a process involving the diploid mechanism of segregation and combination. However, natural polyploidy has been found in annelids, insects, crustaceans, fish and amphibians. In fish, some natural polypoids have been reported (Table 2). The evolutionary significance of this natural occurrence is not yet fully understood.

General Description of Teleost Egg and Sperm

In a typical teleost egg, 44 to 84% of the fresh weight is water, there is a great variation in protein and fat content and the yellowish color is due to soluble carotenoids (Ginzburg, 1972). The number and size of the

Table 2. Naturally occurring polyploid fishes.

Common Name	N[1]	Reference
Atlantic salmon	4	Nygren et al. 1968
Brook trout	3	Allen and Stanley, 1978
California roach	3	Gold and Avise, 1976
Fathead minnow	3	Gold, 1986
Poeciliids	3	Schultz, 1967
Rainbow trout	3	Cuellar and Uyeno, 1972 Thorgaard and Gall, 1979

[1]N = ploidy level of fish

vacuoles increases in subsequent stages of oocyte development. New rows of vacuoles are added from the periphery to the nucleus until vacuoles occupy most of the cytoplasm (Ginzburg, 1972). Yolk granules store nutritive substances (proteins and lipids) and are concentrated in the vegetal pole. In some fish species, including bluegill, yolk granules unite toward the end of oogenesis and form a homogeneous mass (Morgan, 1951).

The egg membrane has two parts: the zona radiata and the outer membrane (Figure 1). The zona radiata protects the egg from external influences and is pierced by thin tubules where water and nutritive substances enter. The outer membrane is sperm impermeable and very sticky. The micropyle at the animal pole is a funnel shaped pit in the membrane that passes into a tubule that opens to the cytoplasm. The diameter of the micropyle corresponds to the width of the sperm head. The cortical alveoli have the function of protecting the egg from penetration by multiple sperm. After fertilization they discharge their contents into the micropylar region and block sperm penetration (Ginzburg, 1972). During ovogenesis, reserve substances (yolk granules and lipids) accumulate in the cell to provide nutrients for the embryo.

Female meiosis provides an example of deviation from symmetric division. Females commit their resources to produce one large egg and the three unused chromosome

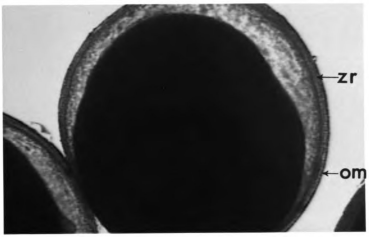

Figure 1. Bluegill egg showing the zona radiata (zr) and outer membrane (om), 30 minutes postfertilization.

complements are discarded in small cells called polar bodies (O'Farrell et al. 1989). A maturation promoting factor (MPF) stimulates the oocyte to enter into meiosis (Murray and Kirschner, 1989). As the nuclear membrane dissolves, the spindle of the first meiotic division forms near the karyosphere which is a small dense body with chromosomes distributed on it. The chromosomes are arranged on the equatorial plane of the spindle and form the metaphase plane. With the separation of the first polar body, the spindle of the second meiotic division appears at metaphase II; meiosis is halted and ovulation occurs (Ginzburg, 1972).

The fish sperm is composed of three parts: spherical head, middle piece and tail. Milt is a suspension of sperm and seminal fluid where the sperm is immobile and needs water for activation. In fresh water fishes, sperm is motile in water for only two to three minutes. There are long and short lived sperm and there is variation in the period of active movement for sperm of the same batch (Ginzburg, 1972).

In teleost fish with external fertilization, the sperm has lost the acrosomal reaction that was once necessary to penetrate the egg membrane. The egg membrane is impermeable to sperm but it has a micropylar system in the animal pole. The sperm penetrates the egg through the micropyle and the nucleus and internal organelles are absorbed by cytoplasm (Ginzburg, 1972). After fertilization, cortical granules

are discharged into the micropylar region and block sperm penetration. Fusion of pronuclei occur after gametic association; the sperm head is transformed into a pronucleus and fuses with the female pronucleus restoring the diploid condition (Ginzburg, 1972). Following a meroblastic cleavage pattern only a portion of the egg divides. The high concentration of yolk impedes the division of the whole egg and the furrows do not extend beyond the blastodisc (Morgan, 1951).

Fish Chromosomes: Numbers and Size

Chromosome numbers in fish cover nearly the whole scope of vertebrate chromosome number, ranging from n=8 in Notobranchius rachowi to n=82 in Ichthymucon gagei (Post, 1973). Peak frequencies of chromosome number are found at n=20 to 28 (80%) and n=40 to 52 (5-6%). Chromosome number for Lepomis macrochirus is reported to be 2n=48 (Roberts, 1964). Apart from microchromosomes in birds and some reptiles, fishes have the smallest chromosomes in vertebrates (Post, 1973). The average length of fish chromosomes is between 2-5 μm, and many species possess numerous small chromosomes of 2 μm or less (Gold, 1979). For Centrarchids, chromosome sizes ranging from 1.2 to 1.8 μm have been reported (Roberts, 1964). Considering the vast number of living fish species, their diversity in morphology and the antiquity of the group as a whole, many orders are

relatively uniform in karyotype (Gold, 1979). However, Roberts (1967) suggested that fish are likely to have more intraspecific chromosome polymorphism than other vertebrates and that karyotypic variation may occur in different individuals of the same species. He also states that the relative similarity of karyotypes in the Centrarchid family with only two chromosome numbers (2n=46 and 48), contrast sharply with the wide range of karyotypes among the Salmonid family. However, even in the Centrarchid family, Lepomis cyanellus is considered to be a typical example of Robertsonian variation (Roberts, 1964). This type of rearrangement may bring changes in chromosome number by fusing two small chromosomes together or dividing a large one and forming two (Gold, 1979).

Karyotype work in fishes has long been impeded by lack of adequate techniques to permit examination of the large number and the small size of their chromosomes (Booke, 1968). Obtaining consistent chromosome spreads of good quality is one of the limiting factors in the study of chromosomes in fishes (Gold, 1979). Throughout the literature, it is obvious that technical difficulties have resulted in reports of chromosome numbers and morphology that are now considered incorrect.

Techniques for Chromosome Observation in Fishes

Several techniques to study cell chromosome in fishes (Table 3) have been used with a wide range of results (McPhail and Jones, 1966; Kligerman and Bloom, 1977). Some of these techniques include tissue sectioning (Nogusa, 1960), air drying cells of different tissues (Chen, 1970; Denton and Howell, 1969; Howell, 1972; Gold, 1974), culturing leukocytes (Labat et al. 1967; Heckman and Brubaker, 1970), culturing fibroblasts (Roberts, 1964; Chen, 1970; Chen, 1975), colchicine injection followed by squashes of different tissues (Wright, 1955; Roberts, 1964; Simon, 1964; McPhail and Jones, 1966) and solid tissue techniques (Kligerman and Bloom, 1977).

Laser Scanning Confocal Microscope

Although the use of light microscopy has been very common in the study of fish chromosomes, the use of laser scanning confocal microscope offers significant promise for advances. The laser scanning confocal microscope is a relatively new instrument that allows the examination of microscope specimens to an unusual depth. In the microscope's name "laser" and "scanning" refer to the method of illumination, "confocal" refers to the method of image formation (Dr. J. Whallon, personal communication).

The term "confocal" indicates that the microscope is aligned so that the illuminated spot and the imaged spot

Table 3. Tissue preparation techniques for chromosome observation in fishes.

Technique	Author
Tissue sectioning	Nogusa, 1960
Air drying cells of different tissues	Denton and Howell, 1969 Chen, 1970 Howell, 1972 Gold, 1974
Culturing leukocytes	Labat et al. 1967 Heckman and Brubaker, 1970
Culturing fibroblasts	Roberts, 1964 Chen, 1970, 1975
Colchicine injection and squashes of different tissues (gonads, gill epithelium and blastodiscs)	Wright, 1955 Roberts, 1964 Simon, 1964 McPhail and Jones, 1966
Solid tissue techniques	Kligerman and Bloom, 1977

coincide precisely. This is not the case in conventional light microscopes, in which the light from out-of-focus areas limits the depth to which a specimen can be examined to just a few microns. The traditional method to try to solve this problem has been to embed the sample in resin or paraffin and then to slice it in thin sections for sequential viewing.

In a confocal instrument, the specimen is scanned with a finely focused laser beam. A pinhole aperture is placed directly in front of the detector at the focal point of light coming from the in-focus part of the specimen. The effect of these modification is to block light from out-of-focus regions. The result of this is an increase in resolution and contrast.

In the process of optical sectioning, the microscope collects a series of images from progressively greater depths in the sample and these images can be stored on the computer hard disk attached to the instrument. These images can also be used to form a three-dimensional composite if this is desirable.

MATERIALS AND METHODS

This study was designed to develop histological techniques to monitor egg developmental stages in order to determine the critical stage for production of triploid and tetraploid bluegill. Secondly, we planned to use these histological techniques to observe and describe differences, if any, between the development of normal eggs and the development of eggs that received pressure or cold shock treatment for the production of triploid and tetraploid bluegill.

Experimental animals

Bluegill ready to spawn were collected by hook-and-line from Lake Lansing (near Haslett, Michigan) and identified using an appropriate reference (Hubbs and Lagler, 1964). The fish were transported to the Michigan State University Aquaculture Center in aerated 163 L coolers. At the Center, they were maintained in a 1900 L circular tank with a water temperature of 22°C +/- 2°C. They were spawned soon after arrival.

Spawning selection

The method used for spawning was described by Rothbard and Pruginin (1975). Females and males previously selected for ripeness were strip-spawned. Ripe fish chosen for stripping must be ready for natural spawning. Ripe bluegills can be easily identified by intense pigmentation (especially males) and a swollen genital area.

Checking for ripeness

Fish were considered ready to spawn when a gentle squeezing of the abdominal area caused the eggs and the milt to flow from the genital pore. Mature eggs have yolk that is slightly granular with a large oil globule. These eggs are perfectly spherical (0.85-1.36 mm), strongly adhesive (Banner and Hyatt, 1975) and have an amber color. Ripe milt has a white color.

Egg fertilization

Dry fertilization was used to prevent premature closing of the micropyle and to enhance the ability of the sperm to penetrate and fertilize the egg. Female bluegills were dried around the genital area and the eggs were expelled into a dry watch glass. Milt was collected from the male using a pipet. Milt and eggs were mixed with a glass rod. When the milt was added to the eggs a stopwatch was started to time egg development. One minute postfertilization, water at 22°C was added to activate the eggs.

Histological techniques to monitor egg developmental stages

Light microscope observations

Experiment I: The use of Bouin's solution as a fixative and the use of carmine stain for whole eggs and blastodiscs at different developmental stages of the fertilized egg

Fertilized eggs were transferred to concave microscope slides using a wide mouth pipet. Samples were fixed by adding Bouin's solution (Table 4) to the slides at 30, 40 or 50 min postfertilization. Fixed samples were submerged in a Coplin jar containing Bouin's solution for at least 24 hr.

Whole eggs were rinsed in distilled water to remove excess Bouin's solution and dipped in a Coplin jar containing carmine for 5, 10 or 15 min. They were sequentially rinsed with distilled water to remove excess stain and dipped in Coplin jars containing 30%, 50%, 70%, 95%, 95%, 100% and 100% EtOH and 100% and 100% xylene for 10, 30 or 60 min for dehydration.

With the use of forceps and under the dissecting microscope, blastodiscs were dissected from whole eggs. The blastodisc membrane was removed before staining and the carmine was applied directly to the blastodiscs for 5, 10 or 15 min. Excess carmine was removed by rinsing the blastodiscs with distilled water. Dehydration was attempted as described for whole eggs.

Slides were prepared by air drying the samples for a few minutes. Three drops of permount were added to the

Table 4. Composition for Bouin's and Farmer's fixatives used to fix fertilized bluegill eggs.

Reagent	Composition	ml
Bouin's Solution	Picric Acid	75
	Formalin	25
	Glacial Acetic Acid	5
Farmer's Solution	95% EtOH	75
	Glacial Acetic Acid	25

slide and a coverslip was placed over the sample. The slides were then placed on a heat plate at 48°C for 24 hr. After this, slides were ready to be examined under the light microscope.

Experiment II: The use of Bouin's solution as fixative for eggs at different developmental stages and the use of conventional histological techniques

Fertilized eggs were transferred to glass vials using a wide mouth pipet. Clumped eggs were dispersed and the samples were fixed by adding Bouin's solution to glass vials. The eggs used in this experiment were fixed with Bouin's solution 30, 40 or 50 min postfertilization. They remained in the solution for at least 24 hr. The conventional histological techniques used in this experiment are explained in Appendix A, Table 5 to 10. Slide preparation was as described in Experiment I.

Laser scanning confocal microscope observation

Experiment III: The use of Bouin's and Farmer's solutions as fixatives and the use of ethidium bromide to stain fertilized eggs at different developmental stages

Fertilized eggs were transferred to glass vials using a wide mouth pipet. Clumped eggs were dispersed and excess water removed. Samples were fixed by adding Farmer's fixative (Table 4) to glass vials. They remained in the

fixative for at least 24 hr. Another set of samples were fixed by adding Bouin's solution. Samples were fixed at 10 min intervals (from 1 min to 60 min postfertilization).

The fertilized eggs were transferred to concave microscope slides and rinsed with distilled water to remove excess Farmer's fixative or Bouin's solution. They were stained with 0.0025% ethidium bromide for 10 to 20 min and then rinsed three times (for 10 min each) in distilled water. Slide preparation was achieved by placing 8 to 10 eggs on a concave microscope slide and adding glycerol or distilled water.

Experiment IV: The use of Farmer's solution as a fixative and the use of acetocarmine to stain fertilized eggs at different developmental stages

The samples used in this experiment were fixed as described in Experiment III using Farmer's fixative. Samples were transferred to concave microscope slides and rinsed with distilled water to remove excess Farmer's fixative. Fixed samples were stained with acetocarmine for 10 to 20 min and rinsed with distilled water.

The acetocarmine stain was prepared by boiling 100 ml of 45% acetic acid and adding 1 g of certified carmine dye. After filtering the dye, more acetic acid was added (to bring to 100 ml) and the solution was placed in a bottle.

Several staining procedures were used including: 1) staining for 5, 10 or 20 min, 2) staining for 30 s, 3) staining for 30 s and applying 95% ethanol and 4)staining for 30 s, applying 95% ethanol and squashing the sample. Slide preparation was as described in Experiment III.

Experiment V: The use of Farmer's solution as a fixative and the use of Feulgen technique to stain fertilized eggs at different developmental stages

The samples used in this experiment were fixed as described in Experiment III using Farmer's fixative. The fertilized eggs were transferred to concave microscope slides and washed with distilled water to remove excess Farmer's fixative. The eggs were stained using the DNA specific Feulgen technique for 10 to 20 min and rinsed with distilled water.

The Feulgen stain was prepared by pouring 200 ml of boiling distilled water over 1 g of basic fuchsin. After filtering the stain, 30 ml of 1 N HCl and 3 g of potassium metabisulfite ($K_2S_2O_5$) were added to the filtrate. The solution was decolorized for 24 hr in a tightly-stoppered bottle in the dark. After this, 0.5 g of carbon (Norit) was added, then filtered through coarse filter paper and stored in the dark. After the stain application, slides were prepared as described in Experiment III.

Comparison between the development of normal eggs and eggs that received pressure or cold shock treatment for the production of triploid and tetraploid bluegill

To achieve the second objective of this study a sequence of photomicrographs of developing normal eggs was produced. These eggs were fixed at 10 min intervals (from 1 min to 60 min postfertilization) with Farmer's fixative and stained for 10 min with 0.0025% ethidium bromide. Comparisons were made between the normal eggs and the eggs that received the induction treatment (Westmaas, 1992). The induction treatments were as follows:

Triploid treatment: Pressure shock of 8000 psi for 5 min applied to eggs at 1.5 min postfertilization. Eggs were fixed at 35, 45 and 55 min postfertilization.

Tetraploid treatment I: Cold shock of 5°C for 10 min applied to eggs at 35, 40 and 45 min postfertilization. Eggs were fixed at 45, 50 and 55 min postfertilization, respectively.

Tetraploid treatment II: Pressure shock of 8000 psi for 5 min applied to eggs at 35, 40 and 45 min postfertilization. Eggs were fixed at 40, 45 and 50 min postfertilization, respectively.

RESULTS

Development of histological techniques to monitor egg developmental stages

The success of polyploid induction techniques depends on the timing of application of the treatment to the developing egg, intensity of the treatment and the duration of exposure for each species. To achieve the first objective of this study, several histological techniques were tested to determine their effectiveness in monitoring the developmental stages of fertilized bluegill eggs. These techniques included standard light microscopy and the use of laser scanning confocal microscopy. The procedures for the preparation of the samples were developed to study chromosomal changes in the nucleus of the blastodisc in the fertilized egg.

Light microscope observations

Experiment I: The use of Bouin's solution as a fixative and the use of carmine stain for whole eggs and blastodiscs at different developmental stages of the fertilized egg

Whole eggs:

Samples fixed with Bouin's solution at 30, 40 and 50 min postfertilization were used. Samples were stained with carmine for 5, 10 and 15 min. There was very little infiltration of the stain in the samples stained for 5 min and too much infiltration in the ones stained for 15 min. From direct observation under the light microscope of the samples stained for 10 min, the micropyle and the blastodisc were easily identified but no other organelles (including the nucleus) were seen.

Blastodisc:

The same staining procedure was used for blastodiscs dissected from whole eggs that were fixed with Bouin's solution. The dehydration procedure could not be completed with the techniques that were available due to difficulty in handling. Furthermore, blastodiscs were damaged due to the effects of surface tension and handling.

Experiment II: The use of Bouin's solution as a fixative for eggs at different developmental stages and the use of conventional histological techniques

Eggs fixed at 50 min postfertilization:

The blastodisc, micropyle, yolk pieces and granular structures (probably oil droplets) were visible only in a few slides of the serial sections examined under the light microscope. No nuclei inside the blastodisc, or

mitochondria or other organelle in the blastodisc cytoplasm were identified. The blastodisc appeared as a elevated area in the animal pole close to the micropyle of the cell.

Eggs fixed at 40 min postfertilization:

The samples were subjected to an increase in dehydration and infiltration time and also the use of terpineol in an attempt to improve paraffin infiltration. From the study of the slides only pieces of blastodiscs and yolk could be identified.

Eggs fixed at 30 min postfertilization:

Although the samples used in this procedure were subjected to an increase in dehydration and infiltration time, the results were very similar to the results of the procedure used for eggs fixed at 50 min postfertilization. Some complete structures like blastodisc, micropyle, yolk and granular structures were observed in the slides, but no other structures could be identified.

Laser scanning confocal microscope

Experiment III: The use of Bouin's and Farmer's solutions as fixatives and the use of ethidium bromide to stain fertilized eggs at different developmental stages

Samples used in this experiment were fixed at 10 min intervals (from 1 to 60 min postfertilization) with Farmer's and Bouin's solutions. They were stained for 10 and 20 min with ethidium bromide and for the slide preparation glycerol

or distilled water was added directly to the samples. It was found that Bouin's solution produces a coarse structure in the cytoplasm and increases the "natural fluorescence" of the cell to a point where it was not possible to distinguish any structures inside the cell. Farmer's solution was a better alternative as a fixative. Staining by adding 0.0025% ethidium bromide for 10 min was found to produce the best image and distilled water kept the samples hydrated better than glycerol. No chromosomes were observed in any of the eggs examined.

Experiment IV: The use of Farmer's solution as a fixative and the use of acetocarmine to stain fertilized eggs at different developmental stages

Samples were stained with acetocarmine for 5, 10 and 20 min. From the study of these slides, it was found that 5 min in the acetocarmine caused too much infiltration of the stain so different staining times were tried. Eggs were stained with acetocarmine for 30 s, with or without 95% ethanol and then some eggs were squashed. From the eggs stained with acetocarmine for 30 s, a fluorescence similar to the one produced by ethidium bromide was observed; but, it was less intense. The use of 95% ethanol did not improve the image. No chromosomes were identified in the squashed blastodiscs or in any of the eggs observed.

Experiment V: The use of Farmer's solution as a fixative and the use of Feulgen technique to stain fertilized eggs at different developmental stages

Although Feulgen stain is specific for DNA, the results obtained were very similar to those observed in Experiments III and IV. The images produced by the stain were satisfactory but inferior to those produced by ethidium bromide. No chromosomes were observed in any of the eggs examined.

Comparison between the development of normal eggs and eggs that received pressure shock treatment for the induction of triploid and tetraploid bluegill

The second objective of this study was to observe and describe differences between the development of normal eggs and eggs that received pressure or cold treatments for the production of triploid and tetraploid bluegill. This was achieved by comparing sequences of photomicrographs of normal eggs and of eggs that received the shock treatments.

Stage I-Fertilization to 30 min postfertilization:

From the sequence of photomicrographs it is apparent that immediately after fertilization the blastodisc appears to constrict at the margin of the animal pole close to the micropyle, forming a white cap (Figure 2). The perivitelline space between the egg membrane and the yolk enlarges and gives way to protoplasmic accumulation in the

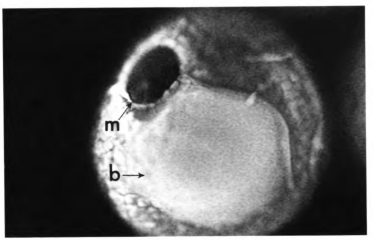

Figure 2. Blastodisc (b) near the micropyle (m) of a normal bluegill egg, 20 minutes postfertilzation.

region (Figure 3). Small oil droplets were observed distributed over the surface of the yolk. After the formation of the blastodisc, no external changes in the fertilized egg were evident until the time of the first mitotic division. With the use of the LSCM, it was possible to measure blastodiscs and micropyles. Blastodiscs diameter ranged from 0.426 mm to 0.523 mm with an average of 0.476 mm (n=11). Micropyle (Figure 4) diameter ranged from 0.191 mm to 0.221 mm with an average of 0.209 mm (n=4). Some polar bodies were observed being extruded from the eggs that received the triploidy induction treatment (Figure 5).

Stage II-First mitotic division:

Following the pattern of meroblastic cleavage in teleost eggs, the first mitotic division occured 35 min postfertilization at 22°C, and the furrow divides the blastodisc into two blastomeres of about equal size (Figure 6). Figures 7 and 8 show the seams in the cleavage furrow and a blastodisc with an incomplete cleavage furrow, respectively.

Stage III-Second mitotic division:

At 50 min postfertilization, it was observed that although the cells had not completed the second mitotic division, four distinct nuclei, two in each blastomere, could be easily recognized (Figure 9). This may indicate that anaphase was probably complete but telophase was yet to

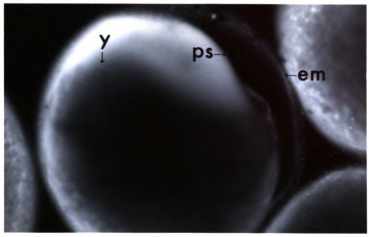

Figure 3. Perivitelline space (ps) between the egg membrane (em) and yolk (y) of a normal bluegill egg, 20 minutes postfertilization, enlarged giving way to protoplasmic accumulation.

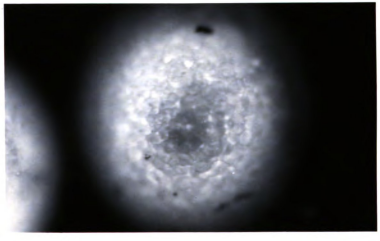

Figure 4. View of the micropyle of a normal bluegill egg, 30 minutes postfertilization.

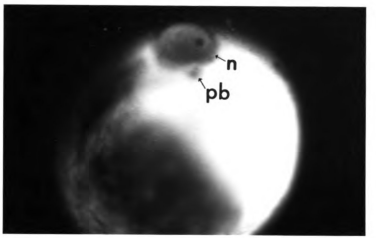

Figure 5. Polar body (pb) being extruded from the nucleus (n) of a bluegill egg, 35 minutes postfertilization, after triploid induction treatment.

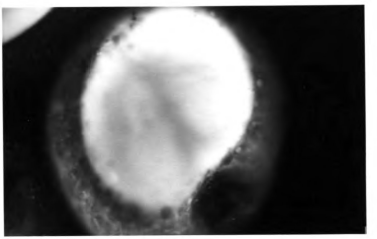

Figure 6. First mitotic division of normal bluegill egg, 35 minutes postfertilization at 22°C, blastodisc is divided into two blastomeres of equal size.

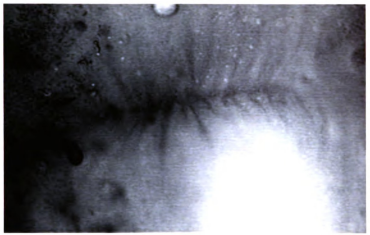

Figure 7. Seams in the cleavage furrow of a normal bluegill egg, 35 minutes postfertilization.

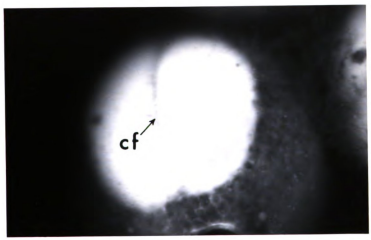

Figure 8. Blastodisc with incomplete cleavage furrow (cf) from a normal bluegill egg, 35 minutes postfertilization.

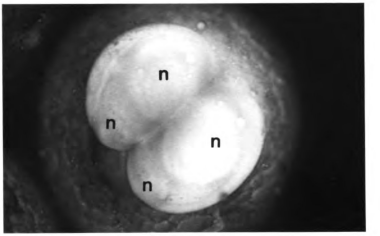

Figure 9. Blastodisc showing two nuclei (n) in each blastomere of a normal bluegill egg, 50 minutes postfertilization.

occur. At 55 min postfertilization the second cleavage furrow appears at a right angle to the first furrow and this resulted in four blastomeres of equal size (Figure 10).

After this stage, cell division becomes more rapid, irregular, and the blastomere size started to become more heterogeneous. Not all the fertilized eggs developed at the same rate, some reached early blastula stage while others were in two or four cell stages.

No dividing blastodiscs were observed in the eggs that received the pressure or cold treatments for the production of triploid and tetraploid bluegill. Blastodics and eggs seemed to be normal in size, shape and general appearance but no cell division had occurred in the blastodiscs of these eggs (Figs. 11, 12, and 13). At least two mitotic divisions were expected since the first mitotic division occurred at 35 min postfertilization and the second at 55 min. The treated eggs were fixed in this same range from 35 to 55 min postfertilization.

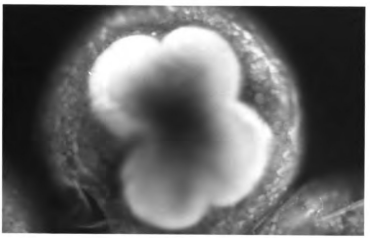

Figure 10. Second mitotic division of a normal bluegill egg, 55 minutes postfertilization, resulting in four blastomeres of equal size.

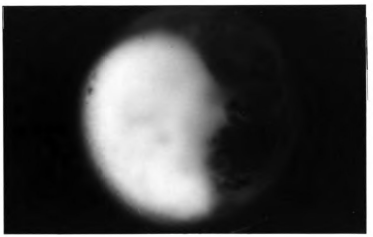

Figure 11. Bluegill egg that received a pressure shock of 8000 psi, triploid induction treatment, 45 minutes postfertilization.

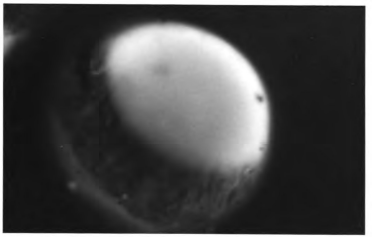

Figure 12. Bluegill egg that received a cold shock of 5°C, tetraploid induction treatment, 55 minutes postfertilization.

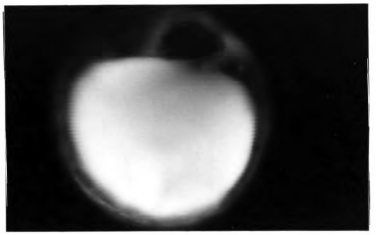

Figure 13. Bluegill egg that received a pressure shock of 8000 psi, tetraploid induction treatment, 40 minutes postfertilization.

DISCUSSION

The bluegill belongs to the Centrarchid family, is the largest lepomine sunfish found in Michigan, the favorite panfish of North American anglers and the most frequently pursued gamefish in the United States (Janssen, 1974). Due to its early sexual maturity and high rate of reproduction, bluegill frequently dominate the biomass of the lakes and ponds they inhabit (Morgan, 1951). Where it predominates numerically, it is prone to stunting (Murnyak et al. 1984). Strategies to overcome this problem have included nongenetic and genetic techniques. Chromosomal manipulation by polyploid induction is one of the most recent genetic techniques proposed as a means of population control in fishes (Bidwell et al. 1985; Myers, 1986; Thompson et al. 1987; Don and Avtalion, 1988).

Several techniques to study cell chromosomes in fishes have been used with a wide range of results (McPhail and Jones, 1966; Kligerman and Bloom, 1977). For the purpose of this study standard histological procedures, light and laser scanning confocal microscopy, were used.

Standard histological techniques and light microscopy

In Experiment I, whole eggs and blastodiscs were fixed with Bouin's solution at 30, 40 and 50 min postfertilization. The samples were stained with carmine for 5, 10 or 15 min. The poor stain infiltration observed in whole eggs may have indicated a permeability problem of the egg membrane. The literature suggests steps that could be taken in order to improve infiltration (Drewry, 1965; Booke, 1968; Gold and Price, 1985). These may include: 1) an increase in dehydration time, 2) the use of different dehydrating chemicals and 3) staining of serial sections of the samples.

The blastodisc dehydration procedure could not be completed due to difficulty in handling. The small size of the blastodiscs made them very difficult to manipulate and the effect of surface tension and handling damaged them.

In Experiment II, Bouin's solution was used as a fixative and conventional histological techniques were applied to fertilized bluegill eggs fixed at 30, 40 and 50 min postfertilization. A possible explanation for not having been able to identify any organelle in the cells may be that the procedures were too harsh for the eggs. The excessive hardening of the egg prevented suitable sectioning by the microtome and only scattered pieces of eggs were recovered on the slides.

Hardening of the blastula tissue prevented effective spread of chromosomes. Drewry (1964) found that fixatives containing alcohol caused hardening of the tissue and prevented suitable examination and squashing. To remedy this situation Booke (1968) suggested the addition of 1 drop of 50% acetic acid to soften the tissue 1 min prior to staining. Also different types of fixatives have been used including methanol-formaldehyde (9:1) which proved to be very effective in preserving tissue (Gold and Price, 1985); however, the use of fixatives containing formaldehyde can increase the natural fluorescence of the cell (Dr. J. Asher, personal communication).

The results obtained indicated that standard histological techniques used in Experiments I and II were not effective in monitoring the developmental stages of fertilized bluegill eggs. If standard techniques are to be used, a change in protocol including the use of different fixatives and chemicals for dehydration and for improved infiltration, and an increase in time for each step is advisable. Disadvantages of these techniques are that they are tedious, time consuming, and there is no guarantee that good quality slides will be achieved.

Roberts (1967) found that some of the disadvantages of using embryonic material to study fish chromosomes were that: 1) in blastomeres the chromosomes tend to be elongated making the counting and determination of the centromere

difficult, 2) the cytoplasm has high affinity for chromosomal stain (due to its high mitochondrial DNA content) and no sharp contrast between the cytoplasm and chromosomes is obtained, 3) in small eggs it is very difficult to separate the blastomere from the yolk, 4) the yolk interferes with staining and obscures mitotic structures and 5) in late embryos the percentage of dividing cells is reduced. One or more of these factors may have interfered with the success of the histological techniques used to monitor chromosomal changes in the developing bluegill eggs.

Laser scanning confocal microscopy

The staining procedures applied included the use of ethidium bromide in Experiment III, acetocarmine in Experiment IV and Feulgen stain in Experiment V. The identification of chromosomes in the developing eggs was not possible with any of the staining procedures.

Although it was not possible to monitor chromosomal changes in the cell, the laser scanning confocal microscope (LSCM) was an excellent instrument to observe developmental changes in fertilized bluegill eggs. With the use of LSCM it was found that blastodiscs and micropyles measured 0.476 mm (n=11) and 0.209 mm (n=4), respectively. With the LSCM it was observed that in normal, fertilized bluegill eggs the first cleavage furrow forms 35 min postfertilization at

22°C. Not all eggs from the same batch developed at the same rate. A number of factors may have affected the rate of meiosis and mitosis in bluegill eggs. Some of these are: 1) the internal temperature of the parental fish, 2) the temperature of the eggs at the moment of fertilization and 3) the incubation temperature of the developing eggs. An increase in any of these temperatures would be expected to produce a faster rate of development.

Although for the purpose of this study the eggs were observed only up to 60 min postfertilization (4 cell stage), Morgan (1951) found that, with an incubation temperature of 22°C, at 1 hr and 40 min postfertilization early blastula was evident and that cells of the blastoderm were quite small. In 3 hr, the marginal cells began to enclose the yolk and by 4 hr the marginal cells had reached the equator of the egg. Between 9 and 12 hr, the yolk was almost completely covered by the marginal cells and an outline of the developing embryo could be seen through the egg membrane.

The LSCM was also a useful tool to study the eggs that received the polyploid induction treatments. No dividing blastodiscs were present in the eggs that were examined. The precise reason for the inhibited cell division in treated eggs is unknown, but it can be argued that the treatment may have produced a shock to the cell system that required a period of recuperation. The various induction

treatments may have interrupted the synthesis of some of the molecules (e.g., proteins and nucleic acids) or the replication of organelles necessary for cell division. The cell may have needed time to "recuperate" before continuing with its normal development.

It was not possible to determine exactly when the first mitotic division occured in the bluegill eggs that were exposed to polyploid induction techniques. The eggs receiving the tetraploid treatments were fixed at 40, 45, 50 and 55 min postfertilization, immediately after receiving the pressure or cold shocks. Eggs receiving the triploid treatments were fixed at 35, 45 and 55 min postfertilization. Cell division had not yet occurred by 55 min postfertilization.

It was also possible that these eggs were polyploid and that, specifically in the case of tetraploid eggs, cell division had been inhibited. However, it is not known whether or not theses cells were polyploid. To determine ploidy level, a study of the chromosome complements would be needed or at least some measure of the DNA content of the cell would have to have been obtained. For the production of tetraploid cells, the induction treatment must be applied prior the first mitotic division of the normal developing zygote (Bungenberg De Jong, 1957; Purdom, 1983; Myers, 1986). For bluegill eggs incubated at 22°C, the first

mitotic division occurred at 35 min postfertilization (Morgan, 1951).

Another possibility for the observed inhibited cell division in eggs that received the polyploid induction treatments could have been that the eggs were dead. Westmaas (1992) observed that the relative survival of bluegill eggs to 4 days posthatching after exposure to the same pressure treatments used to induce triploidy in this study, ranged from 59.4 to 83.7 percent. Relative survival was defined as the ratio of the percent survival of shock treated eggs and control eggs from individual females times 100. Control survival ranged from 76.7 to 87.9 percent (Westmaas, 1992). Consequently, at least some of the eggs exposed to triploid induction treatments should have survived.

Westmaas (1992) also observed that the survival rates of eggs exposed to tetraploid induction treatments were extremely low, usually less than 1 percent. Consequently, few, if any, of the eggs exposed to tetraploid treatments should have been expected to survive.

However, normal eggs quickly change in appearance after they are dead. Dead eggs become cloudy and yellowish in color. These changes were not observed in the eggs that received the induction treatments during the time they were examined.

The analysis of chromosome complements is the most direct assay for ploidy determination (Purdom, 1983). In fish, chromosome analysis can be difficult due to the large number of chromosomes and their small size (Roberts, 1964; Booke, 1968; Gold, 1979). The development of successful methods for observation of fish chromosomes will undoubtedly be beneficial for systematics and for fish phylogeny (Ivanov, 1972), as well as for timing of ploidy induction treatments.

SUMMARY AND CONCLUSION

The objectives of this study were:

1) To develop histological techniques to monitor egg developmental stages to determine the critical time for production of triploid and tetraploid bluegill and
2) To observe and describe differences between the development of normal eggs and eggs that received induction treatments for the production of triploid and tetraploid bluegill.

Several histological techniques were tested to determine their effectiveness in monitoring the developmental stages of fertilized bluegill eggs. These techniques included standard light microscopy and the use of laser scanning confocal microscopy. The procedures for the preparation of the samples were developed to study chromosomal changes in the nucleus of the blastodisc in the fertilized egg.

The results obtained from the standard histological techniques indicated that these techniques were not effective in monitoring the developmental stages of fertilized bluegill eggs. The application of conventional staining techniques to piscine chromosomes was not

effective because chromosomes could not be differentiated from the rest of the cytoplasm. Stains that produce intense differentiation between chromosomal and mitochondrial DNA need to be used. If standard techniques are to be used, a change in protocol including the use of different fixatives, chemicals for dehydration, and techniques to improve infiltration is advisable.

The second objective of this study was partially achieved by comparing sequences of photomicrographs of the development of normal eggs and eggs that received treatment for the production of triploid and tetraploid bluegill (Westmaas, 1992). Although it was not possible to monitor chromosomal changes in the cell and to determine when metaphase of the first mitotic occurred, the laser scanning confocal microscope (LSCM) was an excellent instrument to observe developmental changes in the fertilized bluegill egg.

With the use of LSCM, it was found that blastodiscs and micropyles measured 0.476 mm (n=11) and 0.209 mm (n=4), respectively. The LSCM made it possible to observe polar bodies that were being extruded from some eggs that received the triploidy induction treatment. These polar bodies may have been from the first meiotic division.

With the LSCM, first cleavage furrow was observed at 35 min postfertilization in normal bluegill eggs incubated at 22°C. Consequently, the bluegill eggs that had been shocked

to induce tetraploidy in this experiment were treated after the critical developmental stage to induce tetraploidy.

The LSCM was also a useful tool to study the eggs that received the induction treatments. It was observed that pressure and cold shocks inhibited cell division, as no dividing blastodiscs were present in the eggs that were examined. The precise reason for this is unknown but it can be argued that: 1) the pressure or cold shock treatments may have produced a shock to the cell system that required a period of recuperation, 2) the eggs were polyploid and cell division had been inhibited, and 3) the eggs were dead as a result of the induction treatment.

Observation of fish chromosomes in the fertilized egg may be possible with the use of available techniques (histological techniques or the use of LSCM), but considerable effort will have to be expended in order to identify appropriate chemicals and/or the proper protocols to be used. The development of successful methods for observation of fish chromosomes will undoubtedly be beneficial and will enable researchers to transfer the triploid and tetraploid induction techniques to other gamefish that are prone to stunting like crappie, perch and bullhead.

APPENDIX

Appendix A

Standard histological techniques used to monitor developmental stages in fertilized bluegill eggs

Table 5. Dehydration and paraffin infiltration procedures for bluegill eggs fixed at 50, 40 and 30 min postfertilization.

Stage	Procedure for dehydration and paraffin infiltration
A.	Eggs fixed at 50 min postfertilization
	1. Transfer fertilized eggs into tissue basket
	2. Wash in distilled water to remove excess Bouin's solution
	3. Dehydrate by placing the tissue basket in beakers containing 50 ml of 30%, 50%, 70%, 95%, 95%, 100% and 100% EtOH for 1 hr each
	4. Continue dehydration in two series of 100% xylene for 1 hr each
	5. Place tissue basket into three series of small beakers containing 100% melted paraffin for 1 hr each in the vacuum oven
B.	Eggs fixed at 40 min postfertilization
	1. Repeat steps 1 and 2 as described in A
	2. Dehydrate by placing tissue basket in beakers containing 50 ml of 30%, 50%, 70%, 95%, 95%, 100% and 100% EtOH for 3 hr each
	3. Continue dehydration in two series of 100% xylene for 3 hr each
	4. Place tissue basket into small beakers with different paraffin:terpineol proportions in the vacuum oven (1 hr a to c; 1.5 hr d to f)
	a. 1/4 paraffin: 3/4 terpineol (12.5:37.5ml)
	b. 1/2 paraffin: 1/2 terpineol (25:25ml)
	c 3/4 paraffin: 1/4 terpineol (37.5:12.5ml)
	d. 100% paraffin (50ml)
	e. 100% paraffin (50ml)
	f. 100% paraffin (50ml)

TABLE 5. Continued.

C. Eggs fixed at 30 min postfertilization

1. Repeat steps 1 and 2 as described in A
2. Repeat steps 2 and 3 as described in B
3. Place tissue basket into three series of small beakers containing 100% melted paraffin for 3 hr each in the vacuum oven

Table 6. Paraffin imbedding of bluegill eggs.

Step	Description
1.	Transfer tissue basket from the vacuum oven and open it on the paraffin stove
2.	Deposit melted paraffin into metal boats
3.	Using heated wide mouth pipet, transfer eggs into metal boats
4.	Place plastic square over metal boats and fill slowly with melted paraffin to avoid overflow
5.	Wait at least 24 hr before removing the paraffin blocks from the metal boats

Table 7. Procedure for cutting paraffin blocks with microtome.

Step	Description
1.	To cut the paraffin block using the microtome, place paraffin block (excess paraffin should be removed first) in the microtome and tighten
2.	Clean the blade with xylene (wipe clean one way)
3.	Place the fuzzy side of the blade to the right and tighten the screws
4.	Move the blade close to the block and lock it using the screws
5.	Use 2 brushes to remove the paraffin strips and to keep them away from the blade
6.	Scrape the longer side of the block if necessary to make straight strips
7.	Using a small brush, separate strips for the slides, maintaining proper sequence
8.	Apply albumin to microscope slides and when dry place the paraffin strips on slide
9.	Apply water to the slide using a fine brush (without forming bubbles)
10.	Place on heat plate at 48°C for at least 24 hr.

Table 8. Staining of slides prepared from paraffin blocks.

In this procedure the slides are placed in Coplin jars containing several reagents for different lengths of time:

Reagent	Time
Xylene	4 min
Xylene	4 min
100% EtOH	2 min
95% EtOH	2 min
95% EtOH	2 min
70% EtOH	2 min
Tap water	3 min
Hematoxylin	5 min
Tap water	20 s
Tap water	20 s
Acid alcohol*	1 dip
Tap water	20 s
1% Amn. water**	20 s
Tap water	20 s
Eosin	5 s
D water	1 dip
95% EtOH	1 min
95% EtOH	1 min
100% EtOH	2 min
100% EtOH	2 min
Xylene	3 min
Xylene	3 min
Xylene store for mounting	

*Acid alcohol; 75% EtOH and 1ml HCl
** 1% Amn. water; 99ml water and 1ml ammonia

Table 9. Light microscope instructions (Köhler illumination).

Step	Description
1.	Turn on light
2.	Use low power objetive
3.	Focus in structure (fine focus)
4.	Use next power if desirable and focus again
5.	Close the diaphragm
6.	Focus the condenser until sharp image attained
7.	Center image of the diaphragm with X hair in the center of image (move with screws, they are perpendicular)
8.	Open diaphragm until image fills field of vision (no edge of diaphragm seen)
9.	Remove the ocular and move condenser diaphragm, close 1/2 to 2/3 and put ocular back
10.	Procedure needs to be repeated with every change in power

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