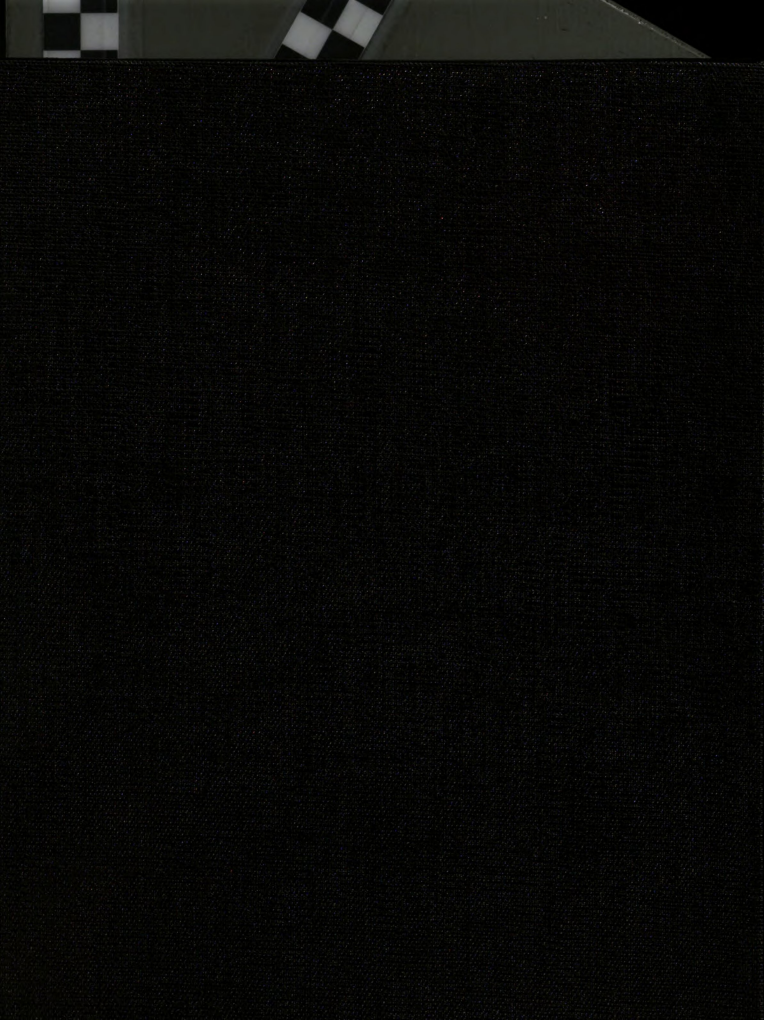




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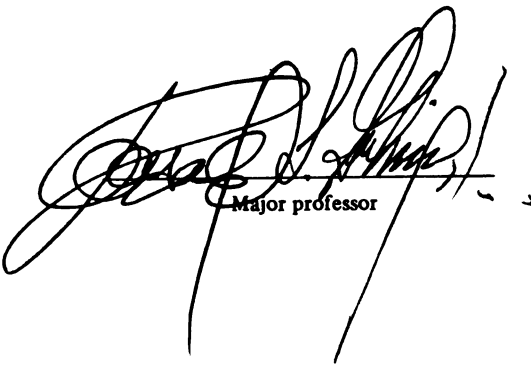
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POLYPLOIDY INDUCTION IN
BLUEGILL SUNFISH (LEPOMIS MACROCHIRUS)
USING COLD AND PRESSURE SHOCKS

By

Andrew R. Westmaas

A THESIS

Submitted to
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ABSTRACT

POLYPLOIDY INDUCTION IN BLUEGILL SUNFISH (Lepomis macrochirus) USING COLD AND PRESSURE SHOCKS

By

Andrew R. Westmaas

697-3863

Triploid and tetraploid bluegill were produced using pressure or cold shocks. Triploidy was induced in all fry tested from pressure shocks (12 replicates) of 8000 psi for 5 minutes beginning 1 to 1.5 minutes post fertilization. Triploidy was induced in 80% of the fry tested from a 6000 psi shock treatment (one replicate). Cold shocks (10 replicates) of 5 C applied for 10 minutes beginning 1 to 1.5 minutes post fertilization induced triploidy in 65.8% of the fry tested. Cold shocks (3 replicates) of 5 C for 5 minutes produced triploidy in 33% of the fry tested. Five fry from each replicate and controls, were tested for ploidy. Modified sample preparation techniques developed in our laboratory enabled us to test the ploidy of fry 7 to 12 days post-hatch using flow cytometry. Triploids produced by pressure had much higher survival (71.6%) relative to controls than triploids produced by cold shock (1.18%).

Two tetraploid bluegill were produced using cold shocks of 5C for 10 minutes beginning 40 and 45 minutes post fertilization. No tetraploids were observed from pressure shock treatments. Fertilized eggs treated with cold and pressure shock 30 or more minutes post-fertilization had very high mortality rates relative to controls.

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KEY TO SCIENTIFIC NOMENCLATURE

<u>Species</u>	<u>Scientific Name</u>
Atlantic salmon	<u>Salmo salar</u> (Linnaeus)
Big head carp	<u>Hypophthalmichthys nobilis</u> (Richardson)
Brook trout	<u>Salvelinus fontinalis</u> (Mitchill)
Bluegill sunfish	<u>Lepomis macrochirus</u> (Rafinesque)
Common carp	<u>Cyprinus Carpio</u> (Linnaeus)
California roach	<u>Hesperoleucus symmetricus</u> (Baird and Girard)
Channel catfish	<u>Ictalurus punctatus</u> (Rafinesque)
Chinook salmon	<u>Oncorhynchus tshawytscha</u> (Walbaum)
Coho salmon	<u>Oncorhynchus kisutch</u> (Walbaum)
Fathead minnow	<u>Pimephales promelas</u> (Rafinesque)
Green Sunfish	<u>Lepomis cyaneus</u> (Rafinesque)
Largemouth bass	<u>Micropterus salmoides</u> (Lacepede)
Rainbow trout	<u>Oncorhynchus mykiss</u> (Miller)
Sailfin molly	<u>Poecilia latipinna</u> (Lesueur)
Shortfin molly	<u>Poecilia mexicana</u> (Steindachner)
Walleye	<u>Stizostedion vitreum</u> (Mitchill)
White crappie	<u>Pomoxis annularis</u> (Rafinesque)

INTRODUCTION

The bluegill sunfish (family Centrarchidae) is one of the most sought after game fish in the United States. Its wide distribution, abundance, flavor, and ease of capture make it very popular among novice and experienced anglers alike (USDI, 1982). In Michigan's Lower Peninsula lakes, the bluegill made up over 50 percent of the biomass in 41 percent of the lakes surveyed, and were the most abundant in 63 percent of these lakes (Schneider, 1981).

The world record hook-and-line bluegill was 4 pounds and 12 ounces (IGFA 1991). Unfortunately, bluegill have a tendency to stunt, especially where they are the most abundant (Swingle, 1950). Stunting has been defined by Burrough and Kennedy (1979) as the exhibition of an individual growth rate well below the potential for the species. Stunted bluegill generally grow to about 3 to 5 inches (Schneider 1981).

Some of the factors that cause stunting include high reproductive potential for the species, low predator densities (Mittlebach, 1981), inappropriate food supply (Murnyak et al. 1984), and an abundance of vegetation in which the bluegill can hide from predators (Carlander, 1977;

The problem is compounded when the already abundant bluegill eat the fry of fish that prey on bluegill such as the largemouth bass (Parker, 1958). Bluegill stunting is an important warmwater fisheries problem facing fisheries managers today. (D.L. Garling, personal communication).

When bluegill are stunted, removal of 80 percent to 95 percent of the biomass will improve the growth rates of the remainder of the bluegill population (Beckman, 1941; Hooper et al. 1964). Methods that have been used to reduce bluegill numbers include seining, poisoning, and disruption of spawning beds (Carlander, 1977). These are temporary solutions to a chronic problem and will only last 3 to 5 years if done properly (Hooper et al. 1964).

Stocking of Lepomine sunfish hybrids has also been suggested as a means of controlling overpopulation in ponds and small lakes. The sex ratios of some lepomine sunfish hybrids are skewed towards males (Childers and Bennett 1961, Childers 1967). However, significant variation in sex ratios of some lepomine hybrids have been observed. For example, the sex ratio of green sunfish ♀ X bluegill ♂ hybrids has ranged from 66 percent to 97 percent (Table 1). Success of controlling overpopulation in ponds using hybrid sunfish depends on sex ratio, amount of vegetative cover, predator population, the presence of parental populations, and fishing pressure (Lewis and Heidinger 1971a).

Table 1. Sex ratios of the green sunfish (GS) X bluegill (BG) hybrids and the reciprocal cross.

Cross ♀ X ♂	State ¹	% ♂	Reference
GS X BG	MI	81	Hubbs and Hubbs (1933)
	MI	87	Laarman (1973)
	IL	97	Childers and Bennett (1961)
	IL	80	Ellison and Heidinger (1978)
	TX	66-78	Crandall and Durocher (1980)
	MS	95	Brunson and Robinette (1987)
BG X GS	IL	64-70	Childers (1967)
	IL	71	Lewis and Heidinger (1971)

¹State where brood stock were collected and crosses were made.

Another potential solution to the stunting problem is to utilize polyploid bluegill in management practices. Polyploid bluegill can be used to control reproductive potential as well as actual numbers within a body of water. Polyploid organisms have three or more sets of chromosomes (Ayala and Kiger, 1984). Normal diploid bluegill have 48 chromosomes (Roberts, 1964). Triploid bluegill would have 72 chromosomes and would be sterile due to the inability of the tetrads to pair during gametogenesis. Tetraploid bluegill would have 96 chromosomes. Tetraploid fishes have viable diploid gametes (Purdom, 1983; Thorgaard, 1986). Possible fisheries applications involving polyploid bluegill include:

1. Triploid fishes may be a desirable management tool for bodies of water susceptible to stunting and overpopulation (Thorgaard and Allen, 1987).

Bodies of water that have been eradicated of stunted bluegill populations, or are simply devoid of bluegill could be stocked with sterile triploid bluegill. With only sterile bluegill in the system, absolute control of numbers is possible, and growth could potentially be maximized. With this scenario, triploid bluegill would have to be stocked annually to maintain optimal densities. Thorgaard (1986) reported that extended growth and survival may be exhibited by mature triploid fishes. This is likely to be true with

bluegill. Because absolute control of bluegill numbers is possible with triploids, aquaculturists may be able to stock grow-out ponds at densities which would allow for maximum growth potential.

2. Tetraploid and diploid bluegill of opposite sexes could be stocked in bodies of water devoid of bluegill (Wilbert, 1990).

Tetraploid bluegill with diploid gametes could be crossed with diploid bluegill with haploid gametes. The resultant fry from this pairing would be triploids (Chourrout et al. 1986). Stocking efforts of proper densities based on estimated survival rates of the parents and offspring would result in an eventual population of sterile triploid offspring produced in that body of water produced over the life of the parental stock.

3. Tetraploid bluegill could be planted into an existing population of diploid bluegill.

The result may be slowed population growth and a gradual shift in the population toward sterile triploid bluegill.

Some of these scenarios have been modeled to predict the potential outcome from these management strategies. These models project bluegill populations in ponds and are

based on several assumptions. These models are presented in Appendix A.

The induction of polyploidy in fishes can be accomplished in a variety of ways (Ihssen et al. 1990). The most common methods have included applying a shock to fertilized eggs at certain stages of development. These methods have included cold shock (Valenti, 1975; Wolters et al. 1981; Cassani and Caton, 1985; Don and Avtalion, 1988a), heat shock (Valenti, 1975; Thorgaard et al. 1981; Chourrout and Quillet, 1982; Cassani and Caton, 1985), pressure shock (Chourrout, 1984), and chemical shock (Refstie et al. 1977; Allen and Stanley, 1979; Smith and Lemoine, 1979; Refstie, 1981; Shelton et al. 1986; Johnstone et al. 1989). Generally, triploidy has been induced in fishes when the appropriate shock was applied just prior to the second meiotic division (Purdom, 1983). Tetraploidy induction shocks have usually been applied just prior to the first mitotic division (cytokinesis) (Purdom, 1983).

The goal of this research was to develop ploidy assessment and polyploidy induction techniques in bluegill sunfish for the purpose of providing fisheries managers with a tool to deter stunting and produce a trophy fishery in small lakes and ponds. The specific objectives of this study were:

1. To develop flow cytometry techniques to measure DNA content in bluegill fry immediately after yolk absorption (age 7 to 12 days).
2. To develop protocols to produce triploid bluegill using cold, and/or pressure shock techniques.
3. To develop protocols produce tetraploid bluegill using cold, and/or pressure shock techniques.

LITERATURE REVIEW

Polyploidy in Fishes

Polyploid organisms have three or more sets of chromosomes. Triploid and tetraploid fish have three and four sets of chromosomes, respectively (Ayala and Kiger, 1984; Chourrout and Nakayama, 1987). Triploid fish are sterile due to the inability of the tetrads to pair during the first meiotic division of gametogenesis. Male triploid fishes of at least some species do undergo the hormonal changes associated with sexual maturation (Benfey et al. 1989) and produce reduced numbers of aneuploid spermatozoa (Van Eenennaam et al. 1990).

In most triploid fishes, gonads are underdeveloped, small, and primarily non-functional (Thorgaard and Gall, 1979; Benfey and Sutterlin, 1984a; Lincoln and Scott, 1984; Johnson et al. 1986). Tetraploid fish are viable and are able to produce diploid gametes (Purdom, 1983; Cassani et al. 1984; Thorgaard, 1986).

Naturally Occurring Polyploids

Naturally occurring polyploids have been observed in a few fish populations. The natural cross of sailfin molly X

shortfin molly resulted in high numbers of triploids (Menzel and Darnell, 1973). Some populations of Cyprinidae have limited numbers of naturally occurring triploids. These include the fathead minnow (Gold, 1986), and the California roach (Gold and Avise, 1976). Naturally occurring triploids have also been found in populations of brook trout (Allen and Stanley, 1978), and rainbow trout (Cuellar and Uyeno, 1972; Thorgaard and Gall, 1979).

Induction of Triploidy

Triploidy induction in fishes requires the inhibition of the second meiotic division of the fertilized egg resulting in the retention of the second polar body. The resultant embryo will have two sets of maternal chromosomes and one set of paternal chromosomes. Any treatment that will prevent formation of or induce depolymerization of the microtubules that make up the spindle apparatus during cell division will prevent the chromosomes from being moved along the centromeres (Reider and Bajer, 1978; Chourrout, 1984). Triploidy has been induced in several fish species using various techniques that are summarized in Table 2.

Survival of triploid fishes is generally lower than controls. In landlocked Atlantic salmon, Benfey and Sutterlin (1984a) reported 70-90% survival relative to controls. Relative survival (survival of triploids ÷ survival of controls) of triploid channel catfish was 88% (Wolters et al. 1981). Survival was significantly lower in

Table 2. Results from selected triploid induction experiments in fishes.

Species	Method	% Triploidy Induction	Primary Author(s) and Year (1900)
Atlantic Salmon	heat	100%	Benfey and Sutterlin 84a
	heat	100%	Johnstone 85
	pressure	100%	Benfey and Sutterlin 84a
common carp	cold	100%	Gervai et al 80
grass carp	cold	18%	Cassoni and Caton 85
	heat	8%	Cassoni and Caton 85
channel catfish	cold	100%	Wolters et al 81
chinook salmon	heat	60%	Utter et al 83
		90%	Westerhof 88
		90%	Spruell 89
rainbow trout	heat	50%	Chourrout 80
	heat	43%	Thorgaard et al 81
	heat	98%	Chourrout and Quillet 82
	pressure	100%	Chourrout 84
white crappie	cold	72-92%	Baldwin et al. 90

production size lots of triploid channel catfish (Wolters et al. 1991). Chourrout et al. (1986) reported lower survival of triploid rainbow trout eggs and larvae relative to full-sib controls. However, survival was similar for triploid and full-sib controls from exogenous feeding through 18 months.

Growth characteristics vary among triploid fishes. Thorgaard (1986), stated that a primary reason for triploidy induction in fishes was their potential for extended growth. Solar et al. (1984) reported inferior growth in young triploid rainbow trout. However, Benfey and Sutterlin (1984a) and Spruell (1989) reported no difference in growth between diploid and triploid salmonids. Triploid catfish were significantly heavier and had better feed conversions than diploids after 8 months of age (Wolters et al. 1982). Further studies of triploid catfish growth in production size lots have indicated that triploid and diploid catfish have equal harvest weights and dress-out percentage, but triploids have significantly lower survival, yield, and higher feed conversions than diploids (Wolters et al. 1991).

Induction of Tetraploidy

The primary interest in tetraploid fishes has been for the production of triploids through the mating of tetraploids with diploids. Tetraploid rainbow trout had viable gametes and were able to reproduce (Chourrout and

Nakayama, 1987). Triploids produced using this method had better survival and performance than triploids produced by conventional shock methods (Chourrout et al. 1986).

Tetraploidy has been induced via the inhibition of the first mitotic division of the fertilized egg and has been induced later as well (Chourrout, 1982; Chourrout, 1984; Refstie 1981; Thorgaard et al. 1981). The treatment has been applied to inhibit the separation of chromatids (karyokinesis), or to inhibit cytoplasmic division (cytokinesis) (Chourrout, 1984). Tetraploidy induction in fishes has proven to be much more difficult to induce than triploidy (Ihssen et al. 1990).

Several attempts have been made to induce tetraploidy in fishes with similar techniques that have been used to induce triploidy (Table 3). Tetraploidy induction success has varied greatly among treatments. Temperature shocks of rainbow trout eggs have yielded 8 to 16 percent tetraploid induction when applied to 36 C for 10 minutes 5 hours after fertilization (Thorgaard et al. 1981). Bidwell et al. (1985) produced 62 percent tetraploids in channel catfish by a temperature shock of 41 C and 3 minute duration and initiated between 80 and 90 minutes post fertilization. Pressure treatments of 7000 psi for 4 minutes beginning 5 hours 50 minutes after fertilization have yielded 100 percent tetraploidy induction in rainbow trout (Chourrout, 1984).

Table 3. Results from selected tetraploid induction experiments in fishes.

Species	Method	% Tetraploidy Induction	Primary Author(s) and Year (1900)
channel catfish	Heat	62%	Bidwell et al. 85
rainbow trout	heat	16%	Thorgaard et al. 81
	heat	8%	Chourrout 82
	pressure	100%	Chourrout 84
tilapia sp.	cold	25%	Don and Avtalion 88b

Survival of tetraploid fishes has been generally low, ranging from 28 to 40 percent. Survival varied by species and induction treatment (Ihssen et al. 1990). Pressure shock treatments of rainbow trout eggs have resulted in 40 percent survival (Chourrout, 1984). Thorgaard et al. (1981) reported 28 percent survival of tetraploid rainbow trout embryos after a heat shock. Bidwell et al. (1985) reported 40 percent survival of tetraploid channel catfish after a heat shock of 40 C.

Growth of tetraploid fishes has been poorly documented. Chourrout et al. (1986) reported very slow growth rates relative to controls (<50%) in rainbow trout when individuals were stocked initially at similar densities in raceways.

Crosses of diploid and tetraploid fish have been attempted in relatively few species. Chourrout et al. (1986) found that the diploid sperm produced by adult male tetraploid rainbow trout had low fertilizing ability compared to diploid adults. Diploid sperm may be larger than haploid sperm and incompatible with the diameter of the micropyle (the opening on the egg that the spermatozoa enters) of the egg. The micropyle on bluegill eggs are extremely large relative to the size of the egg (Montes-Brunner, 1992). Second generation triploid rainbow trout that have been produced by a diploid X tetraploid cross

exhibited better survival and growth than triploids produced by conventional heat shocks (Chourrout et al. 1986).

Ploidy Analysis Techniques

Karyotyping

Karyotyping has long been the standard method for ploidy identification in fishes. This methodology has been used to analyze the chromosomes at metaphase within the nucleus of the cells of the species in question. A simple blood cell staining procedure and preparation technique was developed by Klingerman and Bloom (1977), and has been widely used to identify ploidy level in fishes (Thorgaard et al. 1981; Wolters et al. 1981; Chourrout, 1982; Cassani et al. 1984; Bidwell et al. 1985; Myers, 1986).

Karyotyping, though very accurate when performed correctly, is a time consuming technique. It has been generally very difficult to examine more than a few samples from each individual (Klingerman and Bloom, 1977; Blaxhall, 1983). Other problems associated with karyotyping have included inconsistent smear quality, small chromosomes, and experimenter error (Wattendorf, 1986).

Cytophotometric DNA Measurement

Microdensitometers have been used to determine ploidy by measuring DNA volume in the nucleus cytophotometrically. Feulgen stain is mixed with a blood sample and the DNA content in the erythrocytes is measured with the

microdensiometer (Gervai et al. 1980; Johnstone, 1985).

Cytophotometric DNA measurement has been used to successfully identify ploidy in carp (Gervai et al. 1980), rainbow trout (Shelton et al. 1986) and in triploid Atlantic salmon (Johnstone, 1985; Johnstone et al. 1986).

Comparison of Erythrocyte Volumes

Erythrocyte volume analysis has been extensively used in distinguishing differences between polyploid and diploid fish (Swarup, 1959; Purdom, 1972; Valenti, 1975; Allen and Stanley, 1978; Lemoine and Smith, 1980; Refstie, 1981; Wolters et al. 1982; Lou and Purdom, 1984; Johnstone and Lincoln, 1986). Valenti (1975) calculated erythrocyte volume by the equation:

$$V = (4/3)ab^2$$

where:

a = the major semiaxis (the measure along the center of the longest axis of the cell), and

b = the minor semiaxis of the erythrocyte (the measure across the cell at its widest axis).

Lou and Purdom (1984) measured erythrocytes, cartilage, brain, and epithelial cells and determined erythrocytes were the cell type that produced the most consistent results. Measuring erythrocyte volume was less time consuming than karyotyping and was 92.65 percent accurate in distinguishing

polyploids from diploids in channel catfish (Wolters et al., 1982).

Coulter Counter and Channelyzer

The Coulter counter and channelyzer have been widely used in the identification of ploidy level in fishes. The speed, accuracy, availability, and cost effectiveness of the device has made it one of the primary methods of ploidy determinations (Benfey and Sutterlin, 1984b; Wattendorf, 1986). The Coulter counter has been used to measure the actual erythrocyte volume of the sample. The channelyzer accumulates individual erythrocyte volumes and places them into size intervals. Cell volume differs as ploidy level changes. Ploidy determination is made when a predetermined number of cell volumes have been taken. The mean or median cell volume is recorded and related to ploidy level (Benfey and Sutterlin, 1984b).

Flow Cytometry

Flow cytometry analysis of erythrocyte or tissue DNA in fishes has been widely used by researchers to determine ploidy. It is a fast, accurate, and practical method for determining the ploidy levels of fishes (Thorgaard et al. 1982; Downing et al. 1984). Johnson et al. (1984) compared the flow cytometer to the Coulter counter and channelyzer and found that the flow cytometer was 100 percent accurate

while 11 percent of the Coulter Counter histograms were unreadable.

The flow cytometer is used to measure DNA volume using laser beam technology. Cells are suspended in an aqueous solution, stained with a DNA specific fluorescent stain, and delivered single file via air pressure or vacuum at rates of thousands of cells per second. Each cell is transversed by a laser beam, and the resulting refraction of the beam is filtered and collected by a fluorescent detector. The light signal is converted into an electric signal, measured, digitized, and sent to a computer for analysis, display, and storage (Downing et al. 1984).

The stains that have been typically used in flow cytometry analysis of fish DNA are 4'-6-diamidino-2-phenylindole (Thorgaard et al. 1982; Utter et al. 1983; Solar et al. 1984), propidium iodide (Allen, 1983; Westerhof, 1988; Pine and Anderson, 1990; Wilbert, 1990), and acridine orange (Ewing and Scalet, 1991). Ploidy of rainbow trout (Thorgaard et al. 1982; Solar et al. 1984), Atlantic salmon (Allen, 1983; Graham et al. 1985), coho salmon (Utter et al. 1983; Johnson et al. 1984) Chinook salmon (Westerhof, 1988; Spruell, 1989, Young, 1991), grass carp (Allen et al. 1986), hybrid grass carp X bighead carp (Allen, 1983), white crappie (Baldwin et al. 1990), walleye (Ewing and Scalet, 1991), and bluegill sunfish (Wilbert, 1990) have been analyzed by flow cytometry.

Flow cytometry techniques to test for ploidy have most often utilized erythrocytes as the cell source (Thorgaard et al. 1982; Johnson et al. 1984; Westerhof, 1988; Spruell, 1989; Baldwin et al. 1990; Pine and Anderson, 1990; Wilbert, 1990). Wilbert (1990) tested bluegill erythrocytes (Tables 4 and 5) for ploidy 65 days post-hatching.

Other tissues such as milt or newly hatched fry have been used to identify ploidy (Allen et al. 1986; Cassani et al. 1988; Ewing and Scalet, 1991). Wilbert (1990), suggested that identification of ploidy of bluegill fry shortly after swim up would aid in identifying polyploids that may have otherwise died by the age when enough blood could be drawn for ploidy analysis. Another benefit in determining ploidy of fry may be an increased number of treatments tested and more efficient use of space since fish would not have to be reared to a size where blood samples could be drawn.

Miscellaneous Methods

Other techniques have been used to identify ploidy in fishes. Silver staining has been used to identify triploid fish cells (Phillips et al. 1986). This technique has not been widely accepted as a reliable method because it does not provide absolute identification of polyploid fish. Electrophoresis has been used to determine ploidy levels of

Table 4. Blood sample preparation protocol used by Wilbert (1990) to determine ploidy level of bluegill using flow cytometry. The composition of reagents used in flow cytometric determination of ploidy level in bluegill sunfish are summarized in Table 5.

Step	Description
1.	Blood was drawn from each bluegill using a 25 gauge needle and a 1 ml syringe rinsed with sodium citrate buffer solution (CBS).
1a.	Chinook salmon blood was obtained by a cardiac puncture using a 25 gauge needle and a 1 ml syringe rinsed with CBS. The chinook salmon blood was added directly to the bluegill sample to serve as an internal standard.
2.	Blood from the bluegill and chinook was added to a 12 x 75 mm plastic test tube containing 0.5 ml of CBS by submersing the needle in the buffer and applying slight pressure to the hypodermic. Blood was slowly added until samples were a faint pink color.
3.	Samples were stored on ice or refrigerated until the following steps could be completed.
4.	Samples were centrifuged at 2500 rpm at 10 C for 5 minutes and the supernant was discarded leaving a cell pellet.
5.	Cells were resuspended in 0.5 ml of CBS and vortexed until all clumps disappeared.
6.	Cells were fixed for approximately 15 minutes using 70 percent ethanol. Ethanol was stored on ice prior to use.
7.	Samples were centrifuged as described in Step 4, and the supernant was discarded.
8.	Cells were resuspended in 1.5 ml of propidium iodide solution.
9.	0.5 ml of RNase-A solution was added to each tube.
10.	Samples were run on the Ortho Cytofluorograph.

Table 5. Composition of reagents used in flow cytometric determination of ploidy level in bluegill sunfish.

Reagent	Composition
Citrate Buffer Solution (CBS):	8.55 g sucrose 1.17 g trisodium citrate 100.00 ml distilled water
RNase-A Solution:	1.0 mg RNase-A 5.0 ml Phosphate buffer solution (1X)
Propidium Iodide Solution:	2.5 mg Propidium Iodide 0.5 ml Triton-X 1.85 mg EDTA 50.00 ml Phosphate buffer solution (1X)

Miscellaneous Methods

Other techniques have been used to identify ploidy in fishes. Silver staining has been used to identify triploid fish cells (Phillips et al. 1986). This technique has not been widely accepted as a reliable method because it does not provide absolute identification of polyploid fish. Electrophoresis has been used to determine ploidy levels of grass carp and soft-shell clams (Allen et al. 1982; Wiley and Wike, 1986).

MATERIALS AND METHODS

Experimental Animals

All bluegill used in this study were collected by hook and line from Lake Lansing (Ingham County, Michigan) in June and July of 1990 and 1991. Bluegill caught were identified using key characteristics outlined in Eddy and Underhill, (1978). Males were considered ripe if the milt flowed easily from the genital pore. Ripe eggs from females flowed easily from the genital pore and were clear (Banner and Hyatt, 1975). Ripe fish were placed in a 163 liter cooler filled with lake water. The fish were transferred to the Fisheries Research Laboratory at Michigan State University in East Lansing, Michigan and kept in the 163 liter cooler until needed for polyploidy induction experiments run on the same day.

Laboratory well water from a single source entering the lab at $(12 \pm 1 \text{ C})$ was heated to 26 C by hot water heaters. Heated well water was added to the cooler at approximately 2 liters/minute for a minimum of 30 minutes to acclimate the bluegill to the lab water temperature. The water temperature at Lake Lansing ranged from 22 to 28 C during the study period.

Description of Equipment

Incubation cups

Two part incubation chambers were made from PVC pipe cups that were 5 cm long by 7.62 and 6.35 cm, respectively. Mesh screen (420 mM) was siliconed to one end of each of the pipe and the other ends were left open to form cups. The larger cup was placed over the smaller cup to enclose the eggs in the individual incubation chambers. The incubator cup passed oxygenated water; but, retained the eggs and newly hatched fry (Figure 1).

Cold Shock Unit

A standard household refrigerator with an adjustable thermostat was used to maintain the 5 C shocking temperature for all cold shock experiments. A 38 liter aquarium was placed in the refrigerator and filled with water. A 25.4 cm x 33.02 cm x 6.35 cm pan filled with well water was placed in the top of the aquarium with the rim of the pan resting on the sides of the aquarium. The pan served as the cold shock unit (Figure 2). The water in the aquarium and pan stabilized at 5 C before cold shock experiments began.

Pressure shock unit

The pressure shock unit used in the experiments was based on a design used by Dasgupta (1962). A hydraulic

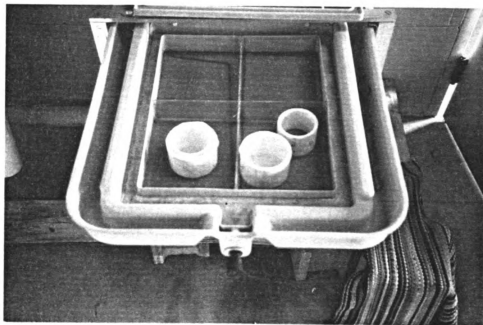


Figure 1. Photograph of the Incubation Cups Used To Incubate Bluegill Eggs in the Polyploidy Induction Experiments.



Figure 2. Photograph of the Cold Shock Unit Used to Treat Fertilized Eggs in the Polyploidy Induction Experiments.

press (F.C. Carver, Inc., New York) with a pressure gauge was used to apply the desired pressure. The press had the capability of building up pressure at the rate of approximately 1000 psi/sec. Decompression speed of the press could be regulated and was nearly instantaneous in the pressure shock experiments.

The treatment vessel of the pressure unit consisted of a cylinder and piston and was constructed by the Physics Department machine shop Michigan State University . The cylinder was stainless steel and was 13.97 cm long with an outer diameter of 5.08 cm and 3.81 cm inner diameter. A 10.8 cm x 1.27 cm stainless steel base was welded onto the bottom of the cylinder for stability. The piston was made of solid brass and was 17.78 cm long. Compression was maintained by two rubber 'O' rings around the base of the piston. A hole was drilled up through the center of the piston and had a release valve at the top which could be opened to allow air to escape and to release the pressure on the apparatus when a treatment was completed (Figure 3).

Spawning Techniques

Female bluegill were rechecked for ripeness before they were utilized in the experiments. Ripe eggs were clear and flowed easily from the genital pore when gentle pressure was applied to the abdomen. Unripe eggs were cloudy and did not flow easily

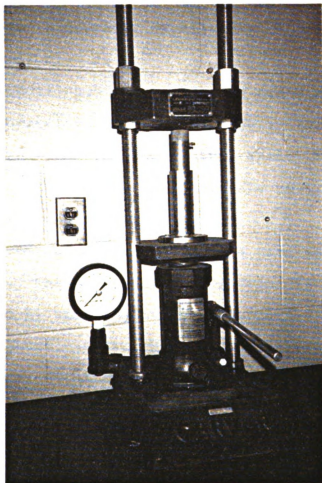


Figure 3. Photograph of the Pressure Shock Unit Used to Treat Fertilized Eggs in the Polyploidy Induction Experiments.

from the genital pore as demonstrated by Banner and Hyatt, (1975). Milt from male bluegill was not checked for motility because milt from at least two males was always utilized. Milt viability from the bluegill was generally not a problem in previous experiments (Wilbert, personal communication).

All bluegill used in polyploidy experiments were hand spawned as described by Wilbert (1990). The hand spawning process began by drying the genital area. Gentle pressure was applied to the sides of the female to expel the eggs onto a dry watch glass.

Milt from two or more males was used to fertilize each set of eggs. The male genital area was dried, and then milt was obtained by applying pressure to both sides of the abdomen (Wilbert, 1990). Milt was collected in a 14.6cm pasteur pipet and pipet bulb (WVR Scientific) and added to the eggs on the watch glass.

The eggs and milt were then stirred gently with a finger to spread the sperm to all eggs (Wilbert, 1990). A stopwatch (Micronta) was started to begin the timing of egg development. Mixing eggs and sperm was arbitrarily chosen as the time of fertilization. At 30 seconds, 26 C water was slowly added to the eggs to water activate the sperm and wash out excess milt (Piper et al. 1983; Wilbert, 1990).

Fertilized eggs that were to be cold shocked were carefully poured into incubator cups just prior to

treatment. Fertilized eggs that were pressure shocked were placed into the incubator cups after the shock treatment was completed. When each replicate was completed, the cups were placed into a Heath incubating unit supplied with 26 C water flowing at approximately 1.5 liters/minute.

Cold Shock Experiments

Cold shock treatments of 5 C temperature have been used to successfully induce triploidy in warm water fishes (Ihssen et al. 1990) and was chosen for the cold shock experiments to induce bluegill triploidy. Fertilized eggs from one female were poured from the watch glass into incubator cups. The cups were placed into the cold shock unit 1 to 1 1/2 minutes postfertilization. The duration of the shock was 10 minutes which has also been successfully used to induce triploidy in other warmwater fishes (Ihssen et al. 1990). Ten experimental replicates with approximately 10,000 to 15,000 eggs per replicate, each with a control, were completed. Control groups consisted of fertilized eggs that were collected and handled in the same way as experimental eggs; but, control eggs were not subjected to temperature shocks.

Tetraploidy experiments using cold shock followed the same protocol as the triploid experiments except shocks were initiated at 20, 30, 40, or 45 minutes post fertilization. Three replicates of each treatment along with controls were

completed for 20, 30, and 40 minute post-fertilization treatments. Only 2 replicates were completed for the 45 minute post-fertilization shock treatment due to a shortage of ripe females.

Pressure Shock Experiments

A sample of approximately 1000 to 2000 fertilized eggs from one female and approximately 15 ml of water were poured into the pressure cylinder just prior to the pressure treatment. Not all of the eggs from a female could be put into the cylinder so a sample of eggs was taken instead. The piston was placed into the cylinder with its valve open and pressed down into the cylinder until water was observed coming out of the valve opening. The valve was shut and the entire unit was placed under the hydraulic press.

Eggs were pressure shocked at 8000 psi beginning 1 to 1.5 minutes after fertilization. The pressure shock was applied for 5 minutes. Twelve replicates and controls of the pressure treatment were run.

Tetraploidy induction experiments using pressure shock were initiated at 20, 25, 30, 35, and 40 minutes post fertilization. The pressure was maintained at 8000 psi for 5 minutes. Three replicates of each treatment were completed along with controls.

Incubation and Rearing

After the eggs hatched any dead, unfertilized, or unhatched eggs were removed from the incubator cups to avoid fungus buildup. In some cases, it was easier to siphon out the fry with a pipet and place them into another incubator cup. Swim up (the life stage when the yolk sac has been absorbed, and individuals begin exogenous feeding) occurred from 6 to 7 days post fertilization when incubated at 26 C (Wilbert, 1990). Fry that were not tested for ploidy within the next 2 to 3 days needed to be fed. These fish were transferred into 38 liter aquaria maintained at 26 C, 0.5 liter/minute flow and equipped with an external stand pipe (Garling and Wilson, 1976). Fry were fed newly hatched brine shrimp as described by Wilbert, (1990). The Photoperiod schedule in the laboratory was maintained at 16 hours of light and 8 hours of dark with throughout the duration of the experiments using an automatic timer (Intermatic Inc.).

Some fry were raised to adulthood in the laboratory. Newly hatched fry were placed in a 38 liter aquaria, and fed newly hatched brine shrimp until they reached approximately 1.27 cm. At this size fry were weaned from the brine shrimp and fed finely ground commercial trout feed (Zeigler, trout grower pellets).

Ploidy Testing

Whole fry were tested for ploidy between 7 and 12 days of age (shortly after yolk absorption) using a new flow cytometry ploidy analysis technique (Table 6). The new technique was based on protocol used to test blood samples as outlined in Tables 4 and 5 (Westerhof, 1988; Spruell, 1989; Wilbert, 1990; Young, 1991). Five fry from each shock treatment replicate and control were tested for ploidy. In some treatment replicates, fewer than five fry survived. All the fry from these replicates were and tested by the flow cytometer.

Ploidy level of the samples was determined on the flow cytometer. An Ortho Diagnostics Systems, Inc. Model 50-h dual laser cytofluorograph and the Ortho Cytofluorograph Analysis for Cellular DNA Content of Fixed Cells with DNA Doublet Discrimination program was used to compile and analyze the data. Bluegill fry samples were run on an argon-ion laser setting of 488 nm with a 0.5 W output.

Pulse-height histograms were generated by either an Ortho 2150 computer or a COMPU-ADD 386 computer with ACQ-CYTE software in conjunction with the cytofluorograph. The histograms were based on DNA volume measurements from 1000 to 2000 cells per sample. Chinook salmon blood was used as an internal standard in each bluegill sample. If histogram peaks shifted, the internal standard helped to discriminate

Table 6. Improved sampling procedure and protocol for testing bluegill fry ploidy using flow cytometry. The composition of reagents used in flow cytometric determination of ploidy level in bluegill sunfish fry are summarized in Table 5.

Step	Description
1.	Place a seven to twelve day old swim up fry into a 12 x 75 mm plastic test tube that contains three drops of citrate buffer solution dispensed from a 14.6 cm pasteur pipet.
2.	Using a glass stirring rod, crush the fry until no large particles are visible.
3.	Add 0.5 ml CBS to the sample tube. Triturate each sample several times using a syringe equipped with a 25 gauge needle.
4.	Add two drops of chinook salmon blood ¹ solution to each sample for use as an internal standard.
4a.	Chinook salmon blood solution is prepared by adding 3 drops of chinook blood to 2 ml of CBS in a test tube and then mixing.
5.	Add 0.5 ml of RNase-A solution to each sample.
6.	Add 1.5 ml of propidium iodide solution.
7.	Centrifuge samples for 5 minutes at 2000 rpm and 10 C.
8.	Remove all but 0.5 ml of supernant and vortex remainder to resuspend samples.
9.	Run samples on the Ortho Cytofluorograph.

¹ Rainbow trout blood or other types of blood containing DNA with histogram peaks generated by flow cytometry that are to the right of the bluegill DNA fluorescence spectrum can be substituted for chinook salmon blood if it is not readily available.

between accidental shifts, and shifts caused by a change in ploidy level.

Survival Study

Most mortalities associated with direct induction of triploidy in fishes occur during initial stages of egg development (Chourrout et al. 1986). Consequently, a study comparing the relative survival of eggs treated with cold and pressure shocks that induced triploidy in bluegill was performed. Survival was estimated at two days post hatching. Three ripe females were used in the experiment. Three males were used per female to insure fertilization.

Each female was first checked for egg quality using the criteria described earlier (Wilbert, 1990; Banner and Hyatt, 1975). To obtain an eggs per gram estimate, two sub-samples of eggs (ranging from 160 to 541) were obtained from each female and counted under a dissecting microscope. Each sample was then weighed to the nearest 0.01 grams. A close approximation of egg numbers for each replicate from each female was obtained. Estimates were a necessary procedure since counting eggs would have been time consuming and egg viability could have been detrimentally affected if the eggs had been exposed to the microscope light, handling, and air during a counting time period.

Egg samples of approximately the same size for two replicates plus a control were obtained from each female and

fertilized with milt from three males. At 1.5 minutes post fertilization, two of the three samples were placed in the cold shock and pressure shock units respectively. The cold shock treatment was administered for 10 minutes at 5 C. The pressure shock treatment was administered for 5 minutes at 8000 psi. The control sample was placed in incubator cups in a Heath incubation tray. After the desired shocks were completed, the samples were placed in incubator chambers and put into the Heath incubator. This procedure was repeated for each female.

Four days after fertilization (two days post-hatching), the newly hatched fry were poured from their respective incubator chambers into a watch glass. The incubation chambers were checked thoroughly for any remaining fry. Individuals were counted using a 14.6 pasteur pipet and bulb. A few fry were removed at a time and placed into another watch glass until all fry from the sample were counted.

For each replicate, percent survival was estimated by dividing the total number of hatched fry counted at two days post hatching by the total estimated number of eggs in the sample and multiplying by 100. Relative survival was calculated by dividing the percent hatch of the respective experimental replicate by the percent hatch of the associated control replicate.

RESULTS

Flow Cytometry Protocol

Flow cytometry was used to identify ploidy of all replicates. Each replicate to be tested for ploidy consisted of the fry that survived to swim-up from the original replicate of fertilized eggs from one female. Five fry were tested for ploidy from each replicate unless fewer than 5 survived. If this was the case, all fry from the respective replicate were tested. The new sampling procedure and staining protocol developed to test the ploidy of 7 to 12 day old bluegill fry (Table 6) was successfully used to differentiate between diploids, triploids, and tetraploids by flow cytometry. The method reduced the time necessary to test ploidy from approximately 2 months to 7-12 days.

Histogram peaks generated by the flow cytometer were a measure of the DNA volume in the of fry tissue cells or chinook salmon blood. The chinook salmon blood peaks used as an internal standard ran from 65 to 70 units of DNA fluorescence and diploid bluegill peaks ranged from 15 to 20 units. Triploid bluegill peaks ran at 1.5 times the diploid peak or 22.5 to 30 units (Figure 4). Tetraploid peaks

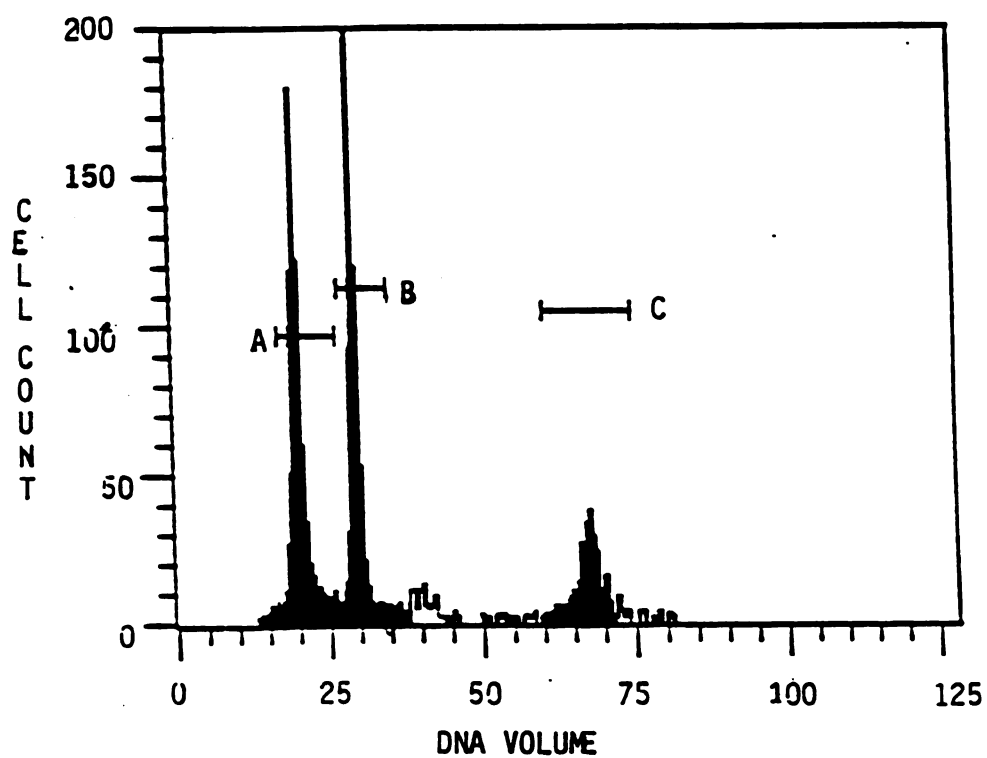


Figure 4. Example histogram peaks generated by ACQ-CYTE software on a COMPU-ADD 386 computer and Ortho cytofluorograph of diploid bluegill fry (A), triploid bluegill fry (B), and diploid chinook salmon blood (C) used as an internal standard.

times the diploid peak or 30 to 40 units of DNA fluorescence (Figure 5). Known diploid bluegill and chinook samples were run at the start of each testing session to set the diploid peak positions. Each testing session had different peak ranges due to the nature of the flow cytometer and its computer. Thus the internal standard (chinook blood) and a known diploid bluegill sample are needed as a reference to measure distance between the bluegill and chinook peaks. This distance remains constant between testing sessions.

Triploidy Induction Experiments

Cold and pressure shock treatments were administered to bluegill eggs at 1 to 1.5 minutes post fertilization for all triploid induction experiments. The cold shock treatments were administered for 5 or 10 minutes at 5 C. Three replicates of the 5 minute cold shock were completed. One replicate produced triploids in all five fry tested. No triploids were produced in the other two replicates (33 percent induction rate). Ten replicates of the 10 minute cold shock treatments produced triploids in 27 out of 41 of the fry tested (65.8 percent induction rate) (Table 7).

Pressure shock treatments were administered for 5 minutes at 6000 to 8000 psi. One 6000 psi 5 minute treatment replicate was completed. Four out of 5 fry tested in this replicate were triploids (80%) (Table 7).

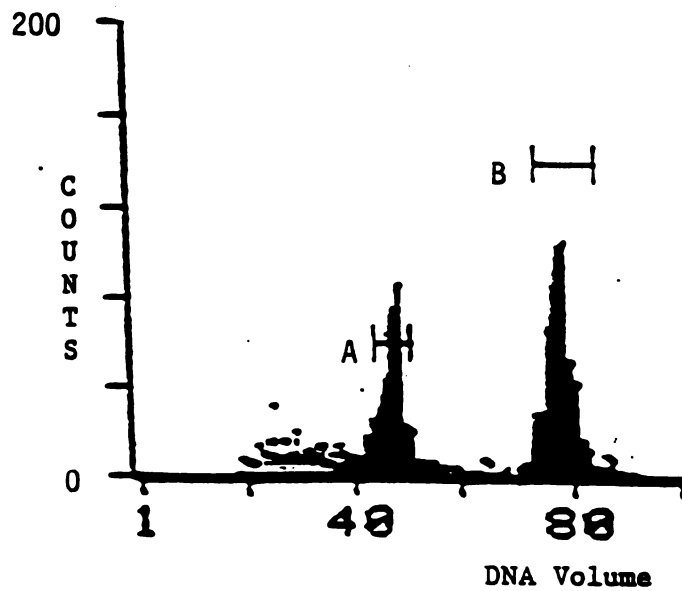


Figure 5. Example histogram peaks generated by Ortho-2150 computer and Ortho cytofluorograph of tetraploid bluegill fry (A) and chinook salmon blood (B) used as an internal standard.

Table 7. Percent induction of triploidy in 4 to 7 day old bluegill fry from polyploidy induction experiments using cold (5C) and pressure shock (6000 psi or 8000 psi) treatments initiated at 1-1.5 minutes post fertilization.

Treatment Type	Shock Length (min.)	Number of Treatment Replicates (n)	Total Number of fry Tested ¹	Percent Triploidy Induction (%)
cold	5	3	15	33
cold	10	10	41	66
6000 psi	5	1	5	80
8000 psi	5	12	60	100

¹ In some instances fewer than 5 fry survived per replicate to be tested for ploidy. In these cases all fry from that replicate were tested.

Twelve replicates of the 5 minute 8000 psi treatment produced triploids in all fry tested (60 out of 60 = 100 percent induction rate).

Tetraploid Induction Experiments

Two tetraploid bluegill were detected from the 12 cold shock replicate groups. A single tetraploid bluegill fry was produced in a 40 and a 45 minute post fertilization cold shock treatment (5 C for 10 minutes). Survival of bluegill from tetraploid cold shock treatments to swim-up was also extremely low (< 5 percent). Six of the 12 cold shock treatment replicates had surviving fry to swim up that could be tested for ploidy (Table 8).

No tetraploid bluegill fry were detected from the 5 pressure treatments (3 replicates per treatment) initiated at 20 to 40 minutes postfertilization. Survival was also extremely low in all tetraploid pressure shock replicates (< 5 percent). Eleven of the 15 attempted pressure shock replicates had fry that hatched. Nine of the 11 replicates had fry surviving to swim up (approximately 6 to 7 days post fertilization) and could be tested (Table 8).

Table 8. Percent induction of tetraploidy in 4 - 7 day old bluegill fry from polyploidy induction experiments using cold (5C) and pressure shock (8000 psi) treatments initiated from 20 to 45 minutes post fertilization (t_0).

Treatment Type	t_0 ¹ (min)	Shock Duration (min)	Number of Treatment Replicates (n)	Total Number Of Fry Tested ²	Tetraploidy Induction (%)
Cold	20	10	3	0	0
	30	10	3	7	0
	40	10	4	20	5
	45	10	2	10	10
Pressure	20	5	3	15	0
	25	5	3	5	0
	30	5	3	3	0
	35	5	3	1	0
	40	5	3	5	0

¹ t_0 = time in minutes post fertilization when shocks were applied

² In some instances fewer than 5 fry survived per replicate to be tested for ploidy. In these cases all fry from that replicate were tested.

Survival Study

Survival of the three control replicates to 2 days post-hatching averaged 83.06 percent. Survival of the cold shocked eggs to hatching relative to controls was extremely low (average = 1.18 percent). One of the three replicates in the pressure shock treatment could not be used because the pressure was inadvertently released midway through the replicate. The average survival of the pressure shocked eggs relative to controls was 71.6 percent (Table 9).

Table 9. Relative survival of bluegill fry to 2 days post-hatching after exposure to cold and pressure treatments initiated at 1 to 1.5 minutes post fertilization.

Female #	Treatment	% Hatch	R.S. ¹
1	control	87.9	100
1	Cold 5 C	0	0
1	Pressure 8000 psi	73.6	83.7
2	control	76.7	100
2	Cold 5 C	1.5	1.9
2	Pressure 8000 psi	45.3	59.4
3	control	85.2	100
3	Cold 5 C	1.4	1.6
3	Pressure 8000 psi	--- ²	--- ²

¹ R.S. = Relative Survival
= (survival of shock treated fish/survival of control fish) X 100

² This replicate could not be used due to inadvertent early release of the pressure.

DISCUSSION

Triploidy was induced in bluegill using pressure and cold shocks. The pressure shock techniques that were developed induced triploidy in all 60 of the fry tested from twelve treatments replicates. Cold shocks were used to induce triploidy in 27 out of the 41 fry tested from 10 treatment replicates. These triploidy induction techniques could probably be applied to other members of the Centrarchid family to induce triploidy. Workers at Southern Illinois University have been successful in inducing triploidy in bluegill X green sunfish and green sunfish X bluegill hybrids by pressure shock. (J. Paret, personal communication). Triploidy has also been induced in white crappie, another Centrarchid, by cold shock treatments (Baldwin et al. 1990).

The high relative survival to two days post hatching (average = 71.6 percent) of triploid bluegill from pressure shock treatments compares favorably with the survival of fish species resulting from pressure shock treatments to induce triploidy. Southern Illinois University workers have had similar relative survival rates with hybrid sunfish (average = 65 to 70 percent) to 5 days post hatching (J.

Paret, personal communication). Relative survival of triploid Atlantic salmon and rainbow trout to hatching produced by pressure shock treatments were 89 percent (Benfey and Sutterlin 1984a) and 53 percent (Chourrout, 1984), respectively.

The poor relative survival of the bluegill eggs exposed to cold shock treatments (average = 1.18 percent) suggests that the treatment may have been too harsh. Valenti (1975) and Wolters et al. (1981) observed much higher survival rates to hatching of tilapia and channel catfish triploids (90 percent and 89 percent, respectively) exposed to cold shocks. The cold shock treatment may have been too harsh in intensity, duration, or both. A reduced cold shock duration or intensity could increase survival while maintaining high triploidy induction rates. Valenti (1975) reported 75 percent triploidy induction in tilapia with 90 percent survival using an 11 C cold shock for one hour. Wolters (1981) however, reported 89 percent survival in channel catfish using a 0 C cold shock for one hour. Baldwin et al. (1990) reported 72 to 92 percent triploidy induction with 5 to 30 percent survival in white crappie (another centrarchid) using a 5 C cold shock for 60 minutes initiated at 5 minutes post fertilization. However, shorter duration cold shock treatments (5 C for 5 minutes) with bluegill were attempted; but, they reduced triploid induction rates from 65.8 percent to 33 percent (Table 7). Warmer treatments of

7.5 C or even 10 C for 10 to 15 minutes may be effective in maintaining high triploid induction rates while producing a higher survival rate.

Two tetraploid bluegill were produced using cold shocks. A single tetraploid bluegill fry was produced in each of two 5 C cold shock treatments for 10 minutes duration initiated at 40 and 45 minutes post-fertilization.

This seems to contradict the notion that tetraploidy is induced just prior to and during the first mitotic division (Thorgaard, 1986) of the fertilized egg which in bluegill occurs on average at 35 minutes post fertilization at 22 C (Morgan, 1951; Montes-Brunner, 1992). Pre-shock acclimation temperatures ranged from 22 C to 26 C. The elevated temperatures that the bluegill were acclimated in may have retarded the rate of egg development as well as the first mitotic division. It is also probable that some late developing eggs were shocked prior to first cleavage at 40 and 45 minutes post fertilization thus producing the tetraploids (Montes-Brunner, 1992). Cold and pressure shocks attempting to induce tetraploidy may delay the time to first cleavage (Montes-Brunner, 1992).

The tetraploidy induction rates observed in this study (10 percent and 5 percent) were somewhat lower than the induction rates produced in other fishes. Thorgaard et al. (1981) reported 16 percent tetraploidy induction in rainbow trout using a heat shock initiated a 5 hours post

fertilization. Chourrout (1982) reported 8 percent tetraploidy induction in rainbow trout using heat shock; but, later reported 100 percent induction in rainbow trout using a pressure shock initiated at first cleavage of 5 hours 50 minutes post fertilization (Chourrout, 1984). Bidwell et al. (1985) reported 62 percent tetraploidy induction in channel catfish using a heat shock initiated around the first cleavage of 90 minutes post fertilization at 27 C. Don and Avtalion (1988b) reported 25 percent tetraploidy induction in tilapia using cold shocks.

No tetraploids were produced using pressure shocks. It is possible that different intensities or durations of pressure shocks could be used to induce tetraploidy in bluegill. The intensity and duration of the pressure shock treatments (8000 psi and 5 minutes) used in this study were based on the assumption that the intensity and duration of the pressure treatment that induced triploidy in bluegill would induce tetraploidy as well. This assumption was based on findings by Thorgaard et al. (1981) who used the same shock temperature (36 C) and duration (1 minute) applied at different initial times to induce triploidy and tetraploidy in rainbow trout.

Extremely low survival was observed in all tetraploid replicates. The low induction rate and low survival of the shocked eggs suggests that some refinements in the

cold and pressure shock durations and intensities should be made. Low survival of tetraploids is common and has been observed by other researchers. Survival of tetraploid rainbow trout to hatching produced by heat shock treatments was 28 percent (Thorgaard et al. 1981); but, was 40 percent for tetraploid trout produced by pressure shock treatments (Chourrout, 1984).

The low survival of tetraploids may have been a result of developmental abnormalities caused by the shock treatment. Myers et al. (1986) suggested that abnormalities may occur in the developing eggs and fry if the shock was applied prior to cytokinesis. Chourrout et al. (1986), noted that abnormal yolk resorption resulting in death was a factor in mortalities of abnormally developing tetraploid rainbow trout; but, also became a factor in apparently normal tetraploid individuals later.

The flow cytometry procedure that was developed to test the ploidy of 7 to 12 day old bluegill fry was easy, fast, and accurate (Table 6). This technique was developed through trial and error and assisted by a series of conversations comparing ideas and experiences with two other researchers (L. King and P. Spruell, personal communication). This technique has also been used successfully to identify ploidy of hybrid sunfish at the facilities at Michigan State University. The technique could probably be used with other Centrarchid fry. It may

also be useful for other fish species whose young are of similar size to bluegill after the yolk has been absorbed.

A similar flow cytometry protocol has recently been developed for larval walleye by Ewing and Scalet (1991). However, this method appears to be more intricate and time consuming.

CONCLUSIONS

The fisheries management and aquaculture potential of triploid bluegill will probably be tested within the next several years. The high survival and 100 percent triploidy induction rate from pressure shock treatments suggests that it is the preferred method to directly induce triploidy in bluegill. Fisheries managers could use pressure shock techniques to make experimental populations of triploids for testing potential in fisheries management situations.

A significant amount of labor is involved in the collection and spawning of large numbers of ripe bluegill. In the Midwest, bluegill spawn from late spring through mid to late summer with peak spawning activities occurring over a 4 to 6 week period. The short window of egg availability coupled with labor intensive collection and spawning techniques may negate the ease of using pressure to induce triploidy directly. The potential use of these techniques to produce large stocks for aquaculture production is currently limited. The low relative survival, difficulty in obtaining ripe eggs, seasonality of egg availability and the expenses incurred to have the fry verified as triploids by

flow cytometry or other methods would probably limit profitability.

Cold shock experiments to induce triploidy produced very few surviving triploids (< 2 percent) and had lower induction rates than pressure shocks (65.8 percent triploids for cold compared to 100 percent for pressure). Cold shock techniques to induce triploidy should be abandoned in favor of pressure shocks.

Production size lots of triploids induced by pressure shock should be attempted. Growth and survival of these fish should be compared to normal diploids in a laboratory. Triploid bluegill fry should also be stocked in ponds at appropriate densities to examine their management potential.

The treatments to induce tetraploidy need further refinement. The appropriate timing of the shock appears to be somewhere between 30 and 50 minutes post fertilization at 26 C. Future experiments need to focus around these times. Various durations and intensities of the shocks should be tested. Future cold shock experiments should use temperatures ranging from 5 C to 10 C, and should vary in duration from approximately 5 to 20 minutes. Several other pressure intensities and durations should be tested at 30 to 50 minutes post fertilization before pressure shock treatments are abandoned.

If tetraploid bluegill stocks could be produced and raised to maturity using improved cold or pressure

techniques, the tetraploid X diploid cross may be used to effectively and economically produce triploids in the future. Other researchers have shown that this cross will produce triploids with higher survival and better overall performance than triploids produced directly by shocks (Chourrout et al. 1986). If tetraploids could be raised to sexual maturity, brood stocks of tetraploids and diploids could be maintained in ponds or at hatcheries. Triploids could be produced from the tetraploid X diploid cross as needed with much less expenditures of time and money.

Our ability to test the ploidy level of young fish may have been the reason why tetraploids were detected. Wilbert (1990) may have induced tetraploidy in bluegill because his heat shock treatments were applied during the appropriate time of first cleavage of the fertilized egg. It is not known if tetraploidy is lethal in bluegill sunfish at later stages of development. If Wilbert (1990) produced tetraploids they may have died during the 65 days needed to obtain sufficient blood volumes for flow cytometry testing. Because the fry in our tetraploidy induction experiments were killed for ploidy analysis, there is no way of knowing if tetraploidy is lethal in bluegill until they can be raised and verified at a later age.

The sampling scheme and staining procedure developed for bluegill fry as part of this study is very fast and accurate. The protocol for this species is probably

applicable for other fishes that produce young of similar size.

APPENDIX

APPENDIX A

Some simple models were developed to test some of the management strategies proposed for polyploid bluegill. Projection tables were used for the models. Age specific survival rate (typically called P_x values), age specific birth rate (called m_x values), length-weight relationships, and carrying capacity estimates were the primary parameters entered into the model. Two different polyploid bluegill management scenarios and a normal diploid population were modeled using a modified version of the Leslie Matrix. For the purposed of simplicity, the following assumptions were made:

1. The theoretical pond for all models is uniform in its characteristics, including;
 - a. The carrying capacity of the pond is approximately 400 pounds per acre. Stunting begins above this level (Garling, personal communication).
 - b. The pond is one acre in size.
2. The populations are closed to immigration or emigration.
3. The growth, fecundity (if applicable), length-weight relationships, and survival rate of normal diploid

bluegill (Krumholz 1947; Beckman 1949) and polyploid bluegill are the same (Table 10). Note this assumption will probably not be true in a real situation but for simplicity was assumed to be true.

4. Male and female bluegill exhibit similar growth and survival characteristics.
5. Bluegill enter the harvestable stock and reach sexual maturity at age four and 6.6 inches.

The management strategies for population control through the use of polyploid bluegill that were modeled were:

1. Sterile triploid bluegill could be stocked in ponds that do not already have existing bluegill populations (new ponds, winter-kill ponds, or renovated ponds). With only sterile bluegill in these bodies of water, control of numbers is possible.
2. Tetraploid and diploid bluegill of opposite sexes could be stocked in bodies of water devoid of bluegill. The matings between these adults would result in a sustained population of sterile triploid bluegill during the reproductive lifetime of the parents.

Table 10. Length-weight relationships, fecundity, and survival of diploid and polyploid bluegill used in the various models.

Age	Survival	Inches	Weight (oz)	Fecundity
0	.1-.4	1.7	.03	---
1	.1-.4	3.1	.30	---
2	.1-.4	4.3	.81	---
3	.1-.5	5.4	1.69	---
4	.4-.6	6.6	3.1	8000
5	.6-.8	7.3	4.21	8000
6	.6-.8	7.7	5.01	8000
7	.6-.8	8.2	6.31	8000
8	.6-.8	8.4	7.0	8000
9	.6-.8	8.7	7.55	8000
10	.6-.8	8.9	7.92	8000
11	0.00	0	0	---

Scenario number one introduces triploids into the pond system and follows the population for 10 years. Since the triploids cannot reproduce, they must be planted every year to obtain a population size that can be maintained near the carrying capacity of the pond. Eight thousand age zero (newly hatched fry) triploids were introduced into the pond the first year. Reducing subsequent plantings over the next several years maintained the population slightly above carrying capacity. The possible ten year population growth curves resulting from planting triploid bluegill into the pond without harvesting and with a harvest is shown in Figure 6. By implementing a harvest on age four fish and older (reducing the P_x value) will maintain the population slightly below carrying capacity. By being able to have absolute control over the number of triploid bluegill in the stock, a fisheries manager can make some predictions as to how many pounds of fish over 6.6 inches the pond owner should harvest every year. The harvest potential at optimal stocking densities in this model reaches approximately 240 pounds in the one acre pond (Figure 7).

Assuming sex can be determined accurately, planting tetraploid and diploid bluegill of opposite sexes into a pond devoid of bluegill is another way that fisheries managers could use polyploid bluegill to control population numbers. This scenario was modeled over fifteen years to show the population trends after the parent bluegill had

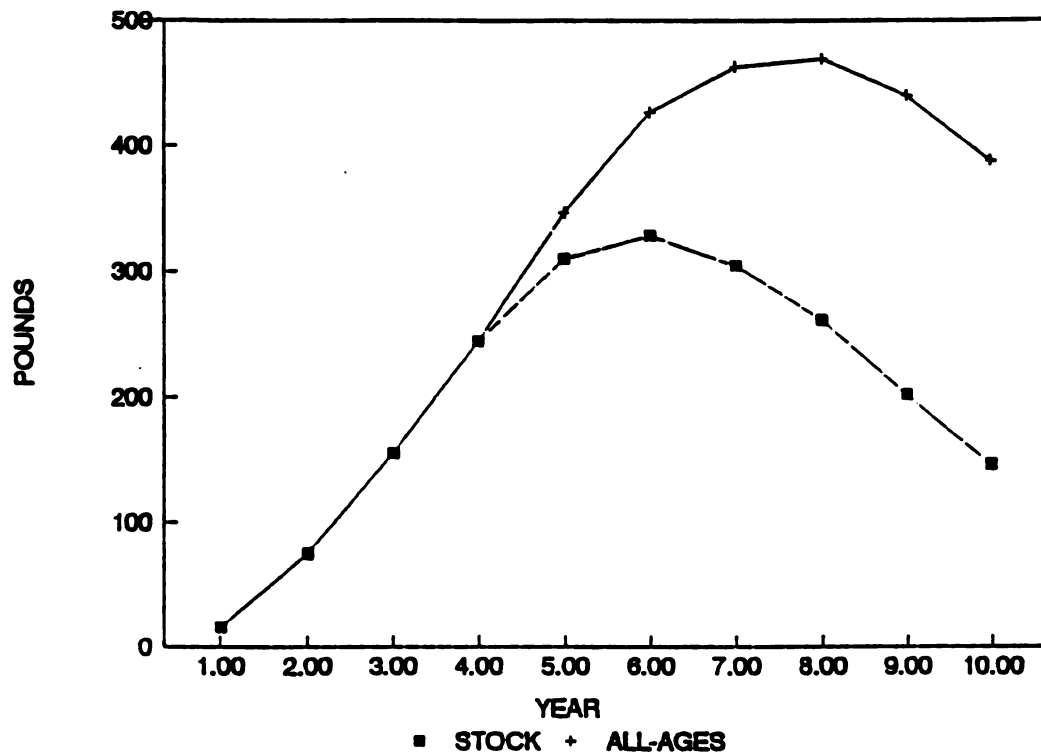


Figure 6. Projected triploid bluegill population growth, with (■) and without (+) harvest, from a one acre pond stocked annually with the triploid bluegill to reach a carrying capacity of approximately 400 pounds.

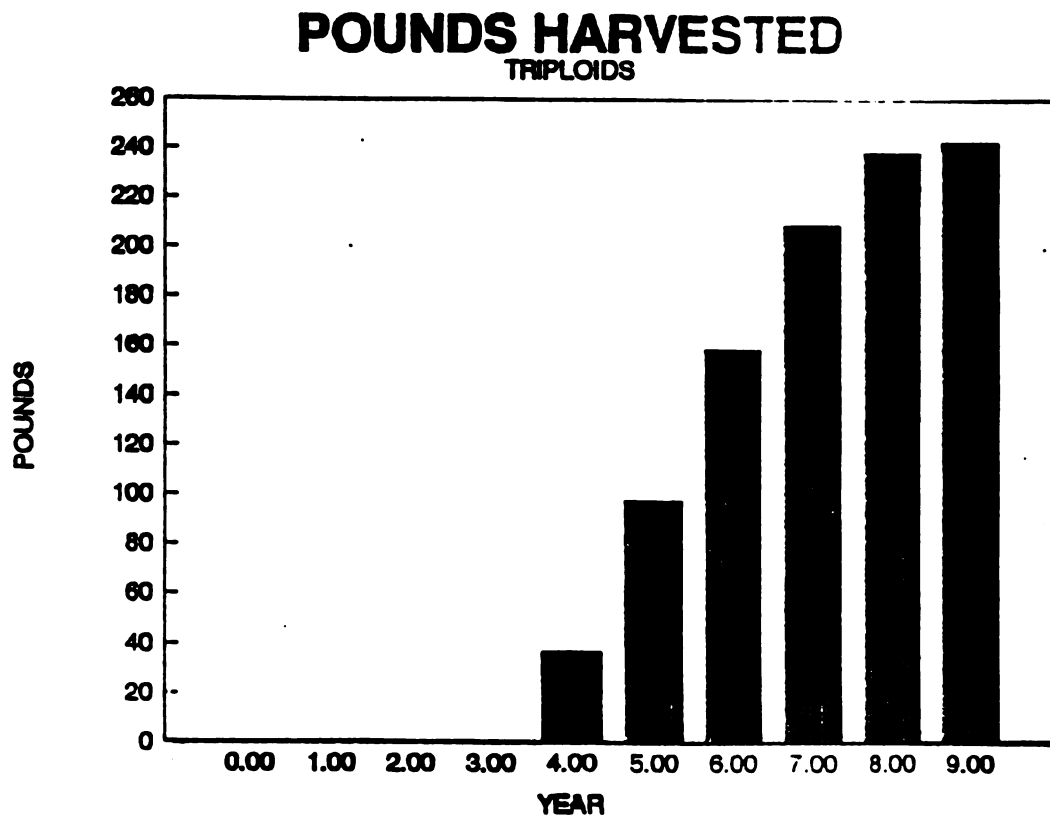


Figure 7. Projected pounds of 6.6 inch triploid bluegill that should be harvested annually from a one acre pond stocked annually with the triploid bluegill to reach a carrying capacity of approximately 400 pounds.

died. To prevent the parent bluegill from being kept when caught by a fisherman, they should be marked with a visible tag. Five adult female diploids and 5 adult male tetraploid bluegill were planted at year one. No other bluegill of any size or ploidy were planted. The fry produced in all years were triploid. The population growth of the triploid progeny in pounds over time with and without a harvest is shown in Figure 8. Figure 9 shows how many triploid bluegill and the pounds of triploid bluegill that should be harvested over the years to maintain the biomass below carrying capacity. It should be noted that further plantings of parent stock at certain intervals should result in a pond that can be maintained near carrying capacity in perpetuity.

For comparative purposes Figure 10 shows what could happen to a diploid bluegill population by planting one female and one male bluegill at year one. This only shows potential because density dependant factors that may limit population growth are not taken into account. Obviously, the population numbers would be greatly reduced with density dependant factors, but the possibility of stunting in bodies of water with the right conditions from this kind of population growth potential is obvious. Note that the population does not take off until year 13, after the fourth generation of bluegill have spawned. In the real world, if conditions were right for stunting in a body of water and

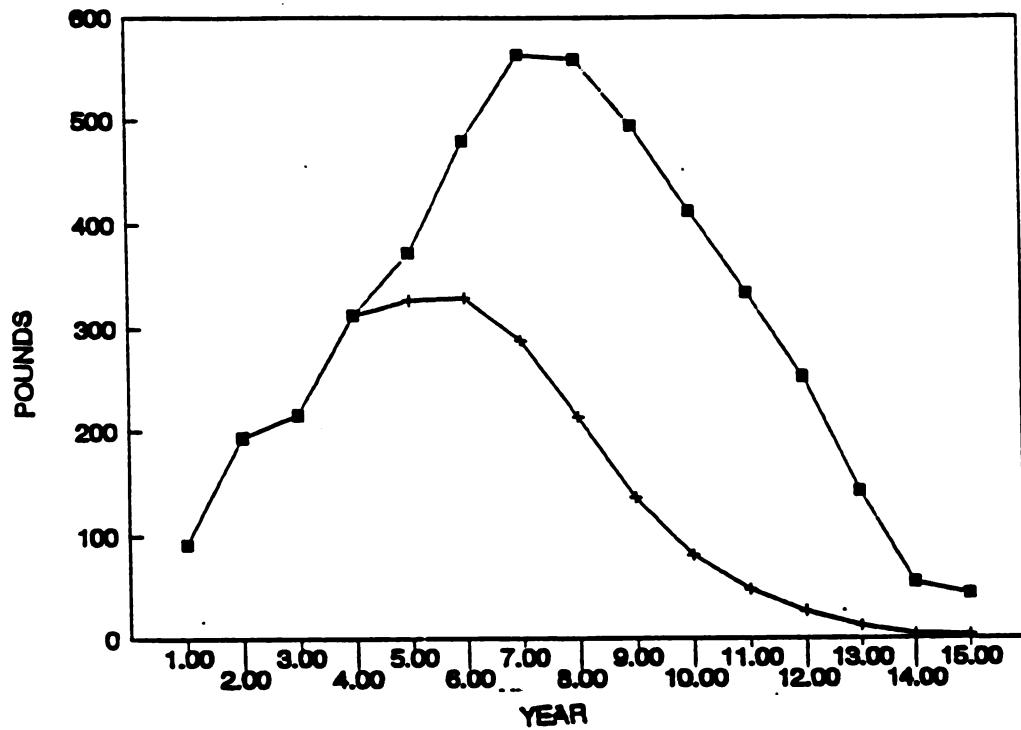


Figure 8. Projected triploid bluegill population growth, with (■) and without (+) harvest, from a one acre pond stocked initially with the 5 diploid and 5 tetraploid bluegill of each sex to reach a carrying capacity of approximately 400 pounds.

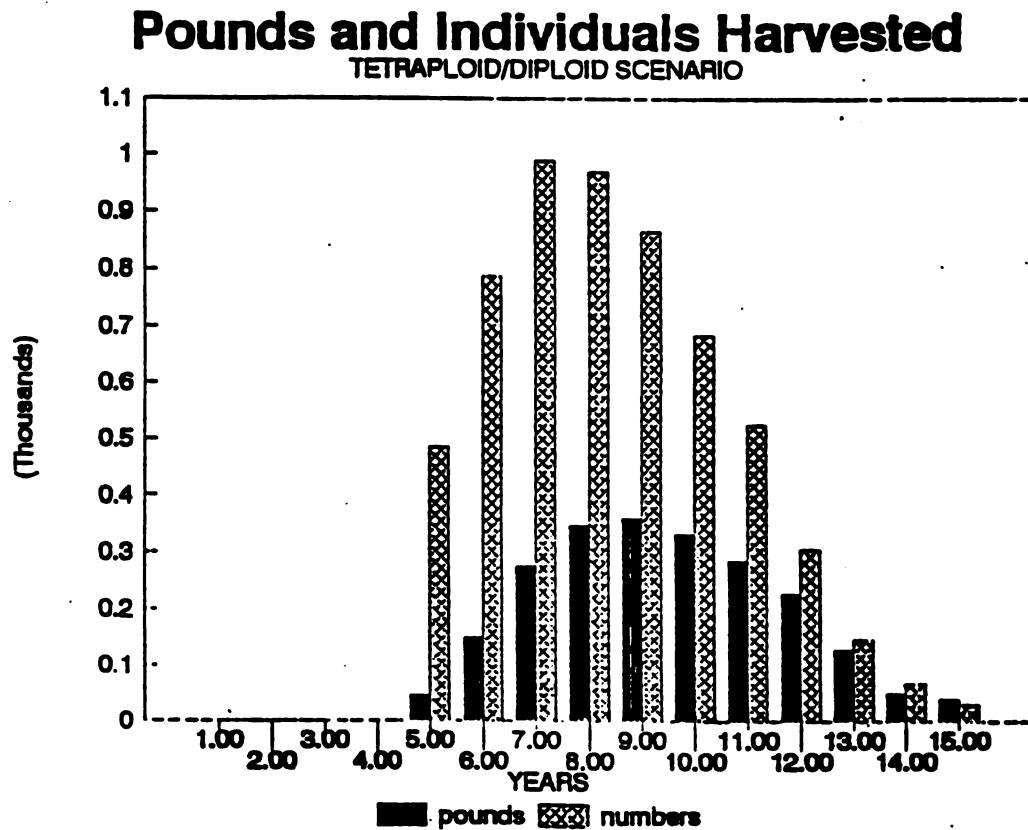


Figure 9. Projected numbers and pounds of triploid bluegill that should be harvested to remain below the carrying capacity of 400 pounds per acre from a one acre pond stocked initially with 5 diploid and 5 tetraploid bluegill of each sex.

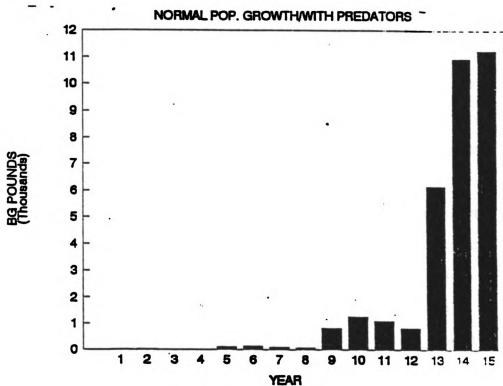


Figure 10.

Projected density independent growth of a diploid bluegill population (pounds) in a one acre pond stocked initially with one male and one female.

bluegill were stocked at typical rates of 200 to 400 young per pond, stunting could occur within 5 years (D. Garling, personal communication).

There are several flaws in the modeling schemes presented as they would apply to a real situation. First, several of the assumptions made would be violated. The fecundity and birth number per female (mx values) generally changes with size and age, but was not taken into account (Carlander 1977). In many cases, ponds are not closed systems. Influences from other fishes or physical factors may cause a substantial change in survival and growth (Smith and Swingle 1943; Krumholz 1947). Also growth and survival of triploid bluegill will probably be substantially different in the fry stages as well as after the normal age of sexual maturity. The modeling schemes presented here can really only be used for comparative purposes within the models. The possible sizes that triploid bluegill could reach at various ages are beyond the scope of these models. However, trends similar to those presented here would be seen if polyploid bluegill were used as a management tool; but much more information would need to be gathered and discovered before accurate predictive models could be used. Some additional information that would make the models more accurate includes: polyploid fecundity, growth, and survival parameters, polyploid spawning behavior, and diploid fecundity at specific ages.

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