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TETRAPLOID INDUCTION PROTOCOLS
FOR BLUEGILL SUNFISH (LEPOMIS MACROCHIRUS)
USING COLD AND PRESSURE SHOCKS

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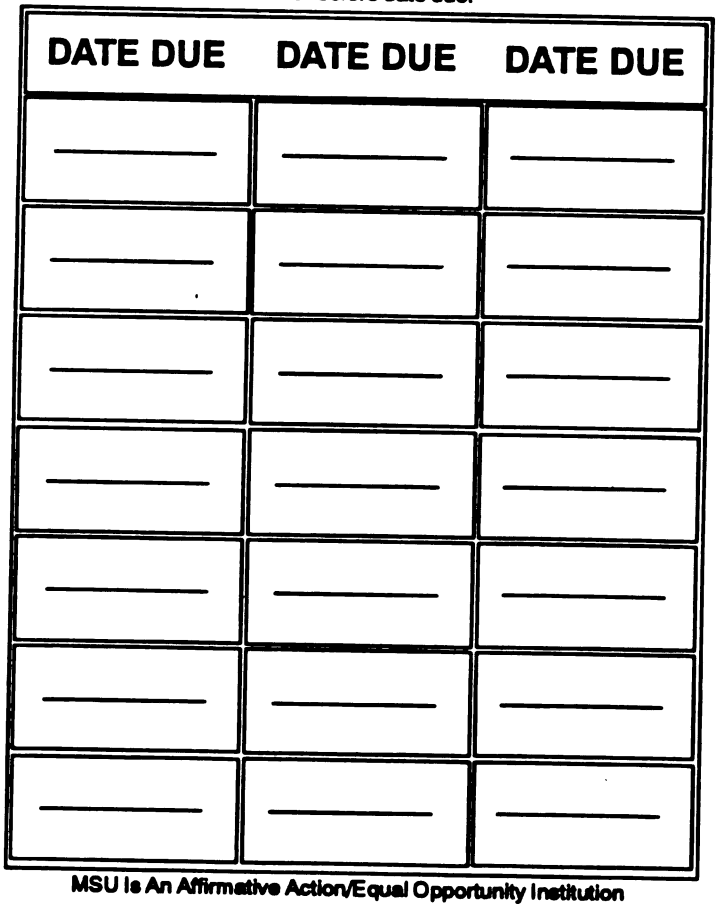

TETRAPLOID INDUCTION PROTOCOLS FOR BLUEGILL SUNFISH (*LEPOMIS MACROCHIRUS*) USING COLD AND PRESSURE SHOCKS

By

Scott Michael Miller

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ABSTRACT

TETRAPLOID INDUCTION PROTOCOLS FOR BLUEGILL SUNFISH (Lepomis macrochirus) USING COLD AND PRESSURE TREATMENTS

By

Scott Michael Miller

Cold and pressure treatments were administered to fertilized bluegill eggs. A total of 16 cold shock treatments were examined. Cold shock treatment parameters ranged from 2.5°C to 10.0°C in intensity, 5 to 20 minutes in duration, and were administered at 35, 40, 45, and 50 minutes post fertilization. A total of 9 pressure shock experiments were examined. Pressure shock treatment parameters ranged from 6,000 to 8,000 PSI in intensity, 10 to 12 minutes in duration, and were administered at 37, 40, 42, 45, and 50 minutes post fertilization.

Whole fry were tested for ploidy between 7 to 12 days of age using flow cytometry. Three of the sixteen cold shocks produced tetraploids. No tetraploids were produced with pressure shock treatments.

Relative survival of cold shock treatments ranged from 0.58% to 33.70%. All 16 cold shock treatments produced fry that survived to be tested. Relative survival of pressure shock treatments ranged from 0.00% to 29.04%. Ten of the 23 pressure shock replicates had fry that hatched and survived to be tested.

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KEY TO SCIENTIFIC NOMENCLATURE

Species	Scientific Name
Amazon molly	*Poecilia formosa*
Atlantic salmon	*Salmo salar*
Big head carp	*Hypophthalmichthys nobilis*
Blue-spotted salamander	*Ambystoma laterale*
Brook trout	*Salvelinus fontinalis*
California roach	*Hesperoleucus symmetricus*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tschawytscha*
Coho salmon	*Oncorhynchus kisutch*
Common carp	*Cyprinus carpio*
Fathead minnow	*Pimephales promelas*
Grass carp	*Ctenopharyngodon idella*
Goldfish	*Carassius auratus*
Jefferson salamander	*Ambystoma jeffersonianum*
Lake trout	*Salvelinus namaycush*
Largemouth bass	*Micropterus salmoides*
Muskellunge	*Esox masquinongy*
Plaice	*Pleuronectes platessa*
Rainbow trout	*Oncorhynchus mykiss*
Silvery salamander	*Ambystoma platineum*

KEY TO SCIENTIFIC NOMENCLATURE CONTINUED

Smallmouth bass	*Micropterus dolomieu*
Tench	*Tinca tinca*
Tilapia	*Oreochromis mossambicus*
Tremblays salamander	*Ambystoma tremblayi*
Walleye	*Stizostedion vitreum*
White crappie	*Pomoxis annularis*
Yellow perch	*Perca flavescens*

INTRODUCTION

The bluegill sunfish (Lepomis macrochirus) has a wide distribution and are abundant throughout North America. Because of their high flesh quality and ease of capture, bluegill sunfish are the most sought after freshwater game fish in the United States (USDI, 1982). Bluegill are very popular among novice and experienced anglers alike.

Despite it's popularity as a sport fish, certain biological factors have lowered the desirability of bluegill in pond management. Stunting is a process that commonly occurs in wild populations of bluegill, especially where they are most abundant. Stunting refers to the inhibition of an individual's growth rate well below the potential for that species (Burrough and Kennedy 1979). According to Schneider (1981), stunted bluegill generally grow to about 3 to 5 inches. Some factors that cause stunting include high reproductive potential for the species, low predator densities (Mittlebach 1981), inappropriate food supply (Murnyak et al. 1984), and overabundance of vegetation which increases escapement (Carlander 1977).

When bluegill are stunted, removal of 80 to 95 percent of the biomass will improve the growth rates of the remainder of the 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 methods are not only expensive and time consuming, but are usually only temporary, lasting 3 to 5 years if performed correctly (Hooper et al. 1964).

Stocking of Lepomine sunfish hybrids has also been suggested as a means of controlling overpopulation in ponds and lakes. The success of hybrid sunfish stocking in eliminating stunting depends on the level of skew in the sex ratio and pond characteristics. The sex ratios of some lepomine sunfish hybrids can be skewed towards males (Childers and Bennet 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 95 percent males (Table 1). Extreme sex ratio variability both within and between crosses leaves this method questionable.

Because of it's popularity as a sport fish, the potential for marketing bluegill is considerable. Many factors make bluegill strong candidates for commercial food fish production (Lewis and Heidinger 1971). Bluegill broodstocks are easy to acquire in high numbers. Spawning of bluegill occurs readily in ponds. Bluegill are not

Table 1. Sex ratios of bluegill (BG) X green sunfish (GS) hybrid crosses.

Cross ♀ X ♂	% Male	State[1]	Reference
GS X BG	80	IL	Ellison and Heidinger (1978)
	66-78	TX	Crandall and Durocher (1987)
	95	MS	Brunson and Robinette (1987)
BG X GS	64-70	IL	Childers (1967)
	71	IL	Lewis and Heidinger (1971)

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

Modified from Westmaas (1992).

inclined to be cannibalistic. Bluegill readily accept pelleted feeds. Bluegill have a high flesh quality.

The development of a bluegill food fish production industry has been impeded, since early sexual maturation in bluegill occurs prior to the attainment of marketable size. This leads to slow and inefficient growth during grow out and uncontrolled reproduction in culture ponds.

One may argue that energy normally spent on growth is shifted to physiological and behavioral changes associated with spawning activity. Physiological changes include development of primary and secondary sexual characteristics such as gonadal development and in the males, color transformation. Behaviorally, bluegill exhibit nest guarding and courtship displays. In addition, uncontrolled reproduction leads to increased population size. Bluegill in extensive culture ponds, with minimal feeding, acquire much of their food resources from natural biota (Garling and Taylor 1984). As the population size increases, competition for food resources increases, resulting in lower growth rates and possibly stunting.

A promising technique and potential solution to the problems caused by early sexual maturation and high levels of reproduction, is the use of polyploid bluegill in pond management and culture practices. Polyploidy refers to the presence of three or more complete sets of chromosomes. Triploid bluegill would have three sets of chromosomes and

would be sterile. Tetraploid bluegill would have four sets of chromosomes and would be fertile (Purdom 1983; Thorgaard 1986).

Production of sterile triploid and fertile tetraploid bluegills could give fisheries managers new tools for the management of lakes and ponds to produce trophy bluegill. Wilbert (1990), suggested several possible fisheries applications involving polyploid bluegill:

1. Tetraploid bluegills could be crossed with normal diploid bluegills to produce sterile triploid bluegills.

2. Sterile triploid bluegills could be stocked alone or in combination with other fishes in ponds or lakes, after sunfish removal, to produce a trophy bluegill fishery. Fish could be stocked at levels that would maximize yield and would have to be restocked annually to compensate for harvest and mortality rates.

3. Diploid and tetraploid bluegill of opposite sexes could be stocked alone or with other fishes to produce triploid bluegill over their life-span. Although less control over bluegill numbers would be possible, the overall number of offspring would be much lower than that from stocking diploid bluegill.

4. Tetraploid or triploid fish could be stocked into existing fish populations. Tetraploids would mate with normal fish and produce sterile triploids. Male triploid bluegill may try to mate with diploid bluegill, but no young would be produced. If tetraploids were stocked periodically, in time, overall bluegill numbers might be reduced and the size of individual bluegills might increase.

Induced triploidy in sunfish could potentially overcome many of the impediments to the development of the aquaculture industry. Past research has indicated that

induced triploidy in other species has resulted in reduced gonadal development, higher vitality, and delayed sexual maturation (Ihssen et al. 1990). If bluegill show similar characteristics, then the problems associated with the onset of sexual maturation before attaining market size such as uncontrolled reproduction and decreased food conversion, would be diminished.

Polyploidy can be induced by inhibiting certain stages of the cell development during embryo formation. Fertilization occurs externally in bluegills, therefore, it is possible to subject fertilized eggs to a variety of physically and chemically induced shocks. Triploidy in fish can be induced by processes that inhibit the second meiotic division of the fertilized egg. Tetraploidy can be induced by any process that inhibits the first mitotic division of the developing egg. Westmaas (1992), examined the use of both cold and pressure shock treatments to induce polyploidy in bluegill sunfish. While both cold and pressure shock treatments succesfully induced triploidy, tetraploid bluegill were produced only with cold shock treatments.

The goal of this research was to improve polyploid induction protocols in bluegill sunfish in an effort to provide both fisheries managers and aquaculturists with tools to enhance bluegill management practices and culture techniques. The specific objectives of this study were:

1. Establish a systematic and complete set of protocols for the production of tetraploid bluegill using cold shock techniques.

2. Establish a systematic and complete set of protocols for the production of tetraploid bluegill using pressure shock techniques.

3. Evaluate the relative survival rate of bluegill fry after cold and pressure treatments.

Literature Review

Polyploidy:

Polyploidy refers to the presence of three or more complete sets of chromosomes. Triploid organisms have three sets of chromosomes and tetraploid organisms have four sets of chromosomes (Ayala and Kiger 1984; Chourrout and Nakayama 1987). Normal bluegill are diploid (two sets of chromosomes). Naturally occurring polyploids are rarely found in animals but are more common among plants. Among salmonids, naturally occurring triploids have been found in rainbow trout (Cuellar and Uyeno, 1972; Thorgaard and Gall, 1979) and brook trout (Allen and Stanley, 1978). In addition to salmonids, 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).

Tetraploidy

Tetraploid fish are of interest for the study of cytogenetic phenomena because polyploidy has played an important role in the evolution of vertebrates (Ohno 1974). Whole families of fish (Catostomidae, Salmonidae) probabley arose as tetraploids (Ohno et al. 1969; Uyeno and Smith 1972). The diploidization of salmonids is still incomplete,

as evidenced by pseudotetrasomy in several species (Wright et al. 1983). Pseudotetrasomy refers to the presence of duplicated gene products.

The primary interest in inducing tetraploidy in fishes is for the production of triploids by mating tetraploids with diploids. To take advatage of the sterility of triploids, it usually is essential to produce pure triplid cultures. For example, exotic triploids are sometimes premitted for an introduction only because of their sterility; even a small incidence of diploid contamination of the triploids could result in the permanent establishment of an udesirable exotic. This also applies to species such as bluegill sunfish and tilapias for which the mass production of triploids by invitro fertilization and physical induction techniques is not practical because of variable induction rates and low survival. Because conventional shock techniques can result in some diploids mixed with triploids, a large amount of time and expense must be spent on analyses to differentiate between them. A more reliable method of inducing triploidy is through the mating of a tetraploid with a diploid, which guarantees the production of 100% triploids.

In addition to the guaranteed production of 100% triploids, the high levels of genetic heterozygosity found in tetraploid gametes - 87% for tetraploids (Diter et al. 1988) versus 56% for gynogenetic diploids (Allendorf et al.

1986) may impart a greater viability to triploids produced from such matings compared with physically induced triploids. Tetraploid rainbow trout had viable gametes and were able to reproduce (Chourrout and Nakayama 1987; Blanc et al. 1993). Chourrout et al. (1986) and Myers and Hershberger (1990) found that diploid sperm produced by tetraploid rainbow trout produced 100% triploids when used to fertilize haploid egg's. However, these crosses resulted in lower fertilization rates relative to diploid X diploid control crosses. Chourrout et al. (1986) further reported that the lower fertilization rates were probably resulted from the incompatibility of the larger diploid sperm with the normal haploid egg's micropyle. The reciprocal cross may have better combining ability (Chourrout ad Nakayama 1987). Second-generation tetraploids have been produced by gynogenesis from tetraploid X diploid matings, and have much better survival than first-generation tetraploids (Chourrout et al. 1986). Also, second-generation triploids produced from such tetraploids had better performance in terms of survival and growth than first-generation triploids or triploids produced by conventional shock methods (Chourrout et al. 1986; Myers and Hershberger 1991).

Tetraploidy is induced by the suppression of the first mitotic division. Treatments have been applied that interrupt karyokinesis, the separation of the chromatids, or cytokinesis, the cytoplasmic division (Chourrout, 1984).

Tetraploidy has proven to be much more difficult to induce in fishes than triploidy (Ihssen et al. 1990).

Variable success has been obtained in tetraploid induction (Table 3). Cold shock treatments of 11°C, initiated at 92 minutes post fertilization, and applied for 1 hour, resulted in 25% tetraploid tilapia (Don and Avtalion, 1988). Bidwell et al. (1985) produced 62 percent tetraploids in channel catfish with a temperature shock of 41°C for a 3 minute duration, initiated between 80 and 90 minutes post fertilization. Thorgaard et al. (1981) produced 16 percent tetraploid rainbow trout with heat shocks of 36°C, applied for 1 minute at 5 hours post fertilization. Pressure treatments of 7000 psi for 4 minutes beginning 5 hours 50 minutes after fertilization have yielded 100 percent tetraploid rainbow trout (Chourrout, 1984). Recoubratsky et al. (1992) also achieved 100% induction of tetraploidy in common carp. Westmaas (1992) used cold shocks of 5.0°C initiated at 45 minutes post fertilization and maintained for 10 minutes to induce 10% tetraploidy in bluegill.

Growth of tetraploid fishes has not been documented to the extent of triploid growth rates. Chourrout et al. (1986) reported very slow growth rates of tetraploid rainbow trout to controls (<50%) when individuals were stocked initially a similar densities in raceways.

Table 2. Summary of results from selected tetraploid (tetra) induction experiments using heat, cold, and pressure shocks; (Relative to controls = rtc).

Species	Induction Method	Result	Reference
Tench	heat	65% tetra, 41% survival to morula	Flajshans et al. (1993)
Tilapia	cold	25% tetra, 16% survival to post hatching	Don and Avtalion (1988)
	pressure	2.9% tetra, inviable after 7 days post fertilization	Meyers (1986)
Channel catfish	heat	62% tetra, 40% survival to hathcing	Bidwell et al. (1985)
Rainbow trout	heat	16% tetra, 28% survival of embryos	Thorgaard et al. (1981)
	pressure	100% tetra, 40% survival to hatching	Chourrout (1984)
Bluegill sunfish	cold	10% tetraploid	Westmaas (1992)

Triploidy:

Triploidy can be induced by any treatment that inhibits 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. Many techniques have been used to induce triploidy on several fish species. Table 2 summarizes the results of using various techniques.

Triploid organisms are typically sterile due to the inability of the tetrads to pair during the first meiotic division of gametogenesis. The resulting gametocytes are non-functional due to the odd chromosome number (aneuploidy). Allen et al. (1986) induced triploid grass carp to spermiation with carp pituitary hormone to determine the chance a euploid sperm could develop from a triploid fish. Euploid refers to having a chromosome number that is a direct multiple of the haploid condition. Euploid sperm could successfully fertilize a normal haploid egg. Flow cytometry revealed the sperm to be 1.5n, 3n, and 6n. The hexaploid cells were considered to be premeiotic unreduced spermatagonia. A high variance of DNA content in 1.5n cells, indicated that the third chromosome set was segregating randomly during meiosis, as would be expected. It was determined that the probability of a euploid sperm being produced was 4×10^{-11} for every meiotic reduction.

Table 3. Summary of results from selected triploid (trip) induction experiments using heat, cold, and pressure shocks; (Relative to controls = rtc).

Species	Induction Method	Result	Reference
Atlantic salmon	heat	100% trip, 90% survival to hatch rtc	Benfey and Sutterlin (1984a)
	heat	100% trip, 67% survival to hatch rtc	Johnstone (1985)
	pressure	100% trip, 89% survival to hatch rtc	Benfey and Sutterlin (1984a)
Common carp	cold	100% trip, 60% survival to hacth rtc	Gervai et al. (1980)
Grass carp	cold	18% trip, 78% survival to blastula	Cassani and Caton (1985)
Channel catfish	cold	100% trip, 89% survival to hatch rtc	Wolters et al. (1981)
Chinook salmon	heat	90% triploid	Westerhof (1988)
	heat	90% triploid	Spruell (1989)
Tilapia	cold	100% trip, 67% survival to hatch	Hussain et al. (1991)
Bluegill sunfish	pressure	100% trip, 72% survival to hatch rtc	Westmaas (1992)
Lepomine Hybrid	pressure	100% trip, 90% survival to hacth rtc	Wills et al. (1994)
Coho salmon	pressure	100% trip, 78.7% survival to hatch rtc	Teskeredzic et al. (1993)
Largemouth bass	pressure	100% trip, 55% survival to prehatch rtc	Garrett et al. (1992)

In addition to possessing non-functional gametocytes, the gonads of triploids are often severely retarded in development. Gonads of triploid Atlantic salmon were reduced in size by 48% for males and 92.3% for females (Benfey and Sutterlin 1984). Jonhson et al. (1986) reported that gonadal development was severely retarded in both sexes of triploid coho salmon at 30 months of age. The average gonadosomatic index (GSI) for triploid coho males was 35.7 percent that of diploids. Gonadosomatic index refers to the growth of the gonads relative to overall body growth at a specific age. The average GSI of triploid coho females was 11.8 percent that of diploid females. Gonad formation was also markedly reduced in triploids of carp, catfish, and plaice (Gervai et al. 1980; Lincoln 1981; Wolters et al. 1981). Researchers at Southern Illinois University have observed a significant reduction in gonads of triploid bluegill x green sunfish hybrids (Sheehan, personal communication). Lincoln and Scott (1984) reported that male rainbow trout exhibited only slightly reduced gonad suppression. Lincoln and Scott (1984) also reported that steroid levels in triploid male rainbow trout did not differ significantly from diploids. However, female triploid rainbow trout had markedly reduced gonad size with low levels of gonadal steroid as compared to diploids.

Although triploid individuals are typically sterile, exceptions to this rule exist in both amphibians and fishes.

However, all of the exceptions are specialized cases which rely on unusual meiotic mechanisms for reproduction. For example, the silvery salamander and the Tremblays salamander are naturally occurring, all-female, triploid hybrids. These hybrids are the result of the mating between the jefferson salamander and the blue-spotted salamander. Silvery and tremblays salamanders reproduce gynogenetically, meaning the sperm from one of the hybrid parental species males only activates the egg to develop. Additionally, the female gametes have no reductional meiotic divisions (Behler and King 1979). A triploid fish species, (Poeciliopsis sp.), also reproduces gynogenetically (Schultz 1967). In this case triploidy is thought to be maintained through an increase in the triploid number early in oogenesis. The triploid number of chromosomes is believed to be increased to hexaploid by an endomitotic division. The triploid number is then maintained by a meiotic division.

Triploids tend to have lower early survival than their full-sib, diploid controls, but survival from the start of exogenous feeding to 18 months was similar to that of controls in rainbow trout (Chourrout et al. 1986). Benfey and Sutterlin (1984a) reported 70-90% survival to hatch, relative to controls in landlocked Atlantic salmon. Westmaas (1992) reported triploid survival rates of 72% to hatching, relative to diploid controls in bluegill sunfish. Wills et al. (1994) estimated survival of triploid hybrid

Lepomis to be at least 90% to hatching, relative to diploid controls.

Growth rates may vary considerably among triploid fishes. Juvenile triploid rainbow trout up to 48 weeks had significantly lower growth rates than diploids (Thorgaard et al. 1982; Solar et al. 1984). However, Chourrout et al. (1986) and Thorgaard (1986) observed that triploid rainbow trout growth exceeded that of diploids after the age of 2 when sexual maturation normally occurs. Spruell (1989) and Wolters et al. (1982b) reported equivalent initial growth rates in chinook salmon and channel catfish, respectively. Higher growth rates were observed after sexual maturity in the triploid channel catfish. Researchers at Southern Illinois University reported similar growth rates between triploid and diploid bluegill x green sunfish hybrids up to a size of 1/4 pound (Sheehan, personal communication). However, dressout weights were significantly higher for triploids than diploids at the same size.

Triploids do not differ from diploids morphologically. Triploid common carp were phenotypically identical to diploids except for a minor disturbance in scale pattern in the triploids (Gervai et al. 1980). Comparisons of multiple morphological measurements, fin ray numbers and pharyngeal teeth arrangement yielded no significant differences between diploid and triploid hybrid grass carp (Cassani et al. 1984).

Gynogenesis

Gynogenesis refers to development in which the embryo contains only maternal chromosomes due to activation of an egg by a sperm that degenerates without fusing with the egg nucleus. It can be induced by stimulating the egg to develop through penetration of inactivated sperm followed by the doubling of the maternal genome. Sperm are inactivated by subjecting them to gamma or ultraviolet radiation. Inactivated sperm contribute no genetic material to the zygote (Purdom 1969). Doubling of the maternal genome has been accomplished by shocking the egg in the same method used for polyploid induction. This prevents extrusion of the second polar body or suppression of the first cleavage, depending on the time of development when the shock is applied. Retention of the second polar body results in some heterozygosity at distant loci due to crossing over. However, total homozygosity results from a suppression of the first cleavage (Ihssen et al, 1990).

There is increasing interest in gynogenesis in fish species in which the male is the heterogametic sex since all female populations could be produced. These populations could be used for the control of reproduction, or the development of inbred lines (Purdom 1983; Chourrout 1982). Inbred lines could be used to study the relative contributions of environmental versus genetic effects (Ihssen et al. 1990).

Androgenesis

In addition to fish with all maternal inheritance, androgenic fish can be produced in which all genetic material is of paternal origin. These fish result when eggs are irradiated by gamma radiation to inactivate the maternal genome (Parsons and Thorgaard 1984). After fertilization with normal sperm, first cleavage is blocked to induce diploidy (Parsons and Thorgaard 1985). Androgenetic diploids could be used to study the phenotypic effects of cytoplasmic constituents such as the mitochondria (Thorgaard 1986). In addition, androgenesis could be used to develop inbred lines and for the recovery of fish lines from cryopreserved sperm (Thorgaard, 1986).

Triploid Hybrids

Hybrids between closely related species may have better growth rates than either parent and can be sterile. Survival of a triploid hybrid may be better than the survival of the corresponding diploid hybrid (Scheerer and Thorgaard 1983). In an ordinary diploid hybrid, some vital hereditary material may be absent because only a haploid set of chromosomes originates from each parental species. Triploid hybrids have three potential aquaculture and fisheries management applications:

1. Higher survival rates of triploid hybrids may increase the feasibility of using hybrid fish that typically have low survival in the diploid condition (Refstie et al. 1982).

2. In species in which hybridization results in sterile offspring, combining triploidy and hybridization offers two forms of sterilization for added security against reproduction (Thorgaard and Allen 1987).

3. It may be possible to combine the desirable traits of two species in their hybrid offspring (Utter et al., 1983).

Ploidy Analysis Techniques

Karyotyping

Karyotyping has been widely used to determine ploidy levels of fishes. A karyotype is the characterization and analysis of a chromosome complement at metaphase within the nucleus of a given cell (Blaxhall 1983). Techniques developed by Klingerman and Bloom (1977), have been used to analyze ploidy of several fishes which have included: rainbow trout (Thorgaard et al. 1981; Chourrout 1982), channel catfish (Wolters et al. 1981; Bidwell et al. 1985), triploid hybrid grass carp (Cassani et al. 1984), triploid chinook salmon (Sweet 1986), and various tilapia species (Myers 1986).

Although karyotyping was extremely accurate when performed correctly, it was also very time consuming. Klingerman and Bloom (1977) and Blaxhall (1983) reported difficulties in examining more than a few samples from each individual. Sweet (1986) observed high variability in chromosome counts. In addition, karyotyping often produced

inconsistent smear quality and small chromosomes were difficult to analyze (Wattendorf 1986; Sweet 1986).

Cytophotometric DNA Measurement

Microdensiometers have been used to determine ploidy levels by measuring nuclear volume cytophotometrically. Nuclear volume of erythrocytes has been measured with a microdensiometer after mixing feulgen stain into a blood sample (Gervai et al., 1980; Johnstone, 1985). Ploidy of several fishes including triploid carp (Gervai et al. 1980), rainbow trout (Shelton et al. 1986), and atlantic salmon (Johnstone 1985; Johnstone and Lincoln 1986) have been assessed using this method. Although nuclear volume estimates by the microdensiometer are very accurate, analysis must be performed on individual cells and analysis of each cell requires a significant amount of time. Without the analysis of large numbers of cells, it is possible to misclassify individuals who may be mosaic.

Comparison of Erythrocyte Volumes

Based on the assumption that polyploid cells are larger than diploid cells, erythrocyte volume analysis has been extensively used in distinguishing ploidy levels in several fish species (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). Microscopic evaluation of erythrocyte volume has been determined by the equation $V = (4/3)ab^2$, were (a) equalled the major semiaxis (the measure along the center of the longest axis of the cell) and (b) equalled the minor semiaxis (the measure across the cell at its widest axis) of the erythrocyte (Valenti 1975). Purdom (1983) measured several cell types (erythrocytes, cartilage cells, brain cells, and epithelial cells) using a microscope and concluded that erythrocytes were the cell type that yielded the most consistent results. Wolters et al. (1982) found that measuring erythrocyte volume was less time consuming than karyotyping. However, erythrocyte analysis was only 92.65 percent accurate and therefore misclassified 7.35 percent of the cells.

Coulter Counter and Channelyzer

In an effort to improve the accuracy and speed of estimating cell volumes, the coulter counter and channelyzer was developed. The coulter counter has been used to measure erythrocyte volumes. Cell volume differs as ploidy level changes. The Channelyzer accumulates individual erythrocytes into size intervals (Benfey and Sutterlin 1984). Ploidy levels have been calculated from mean and median erythrocyte volumes. This method has failed to identify mosaics that have been identified using other methods (Benfey et al. 1984). A mosaic organism has cells

of various ploidy level (eg, both diploid and tetraploid cells). Johnson et al. (1984) used triploid chinook and coho salmon verified by the flow cytometer to determine the accuracy of the Coulter Counter with Coulter Channelyzer and found that 11 percent of the Coulter Counter histograms were inconclusive.

Triploid grass carp are used extensively in North America as a biological control agent for nuisance aquatic plants (Cassani and Caton 1985). The current method used to screen for ploidy is the Coulter Counter. The use of this method may, therefore, result in the misclassification of ploidy levels in grass carp. Johnson et al. (1984) recommends the use of flow cytometry in instances such as the release of grass carp, which are an exotic species and complete triploid groups are required.

Flow Cytometry

Measurement of erythrocyte nucleic acid content by flow cytometry has been widely used and accepted for its accurate and rapid determination of ploidy level in fish (Thorgaard et al. 1982; Downing et al. 1984). Flow cytometry measures and analyzes signals that result as particles flow in a liquid stream through a beam of light. Cells are one type of particle that can be analyzed by flow cytometry. Cells used for ploidy analysis are suspended in an aqueous solution, stained with a fluorescent die specifically

designed for nucleic acids, and delivered single file to a detection center via vacuum or air pressure at rates of thousands of cells per second. Measurements are quantitative when nucleic acids are entirely stained and occurs when sufficient amounts of stain are used. Each cell is transversed by a laser beam, and the resulting refraction of the beam is filtered and converted into an electrical signal, measured, digitized, and sent to a computer for analysis, display, and storage (Downing et al. 1984).

Several stains have been used in flow cytometry analysis of fish cell nucleic acid content. Nucleic acid stains such as propidium iodide are specific to double stranded nucleic acids, while acridine orange stains all nucleic acids. Further treatment of the cells with RNAase ensures that any fluorescense results from the DNA content and not the RNA content of the cell. Thorgaard et al. (1982) and Utter et al. (1983) used 4'-6-daimidino-2 phenylindole. Propidium iodide, (Allen 1983; Westerhof 1988; Pine and Anderson 1990; Wilbert 1990; Westmaas 1992) and acridine orange (Ewing and Scalet 1991) have also been used. 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), bluegill sunfish (Wilbert 1990; Westmaas 1992), and bluegill x green sunfish hybrids (Wills et al. 1994) 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). Milt has been used to identify tetraploid and diploid rainbow trout (Allen et al. 1986; Cassani et al. 1988) and newly hatched fry have been used to indentify ploidy level of walleye (Ewing and Scalet 1991). Westmaas (1992) developed and used a flow cytometry technique to analyze bluegill sunfish fry. Analysis was performed on fry shortly after swim up, once the yolk sac had been absorbed. Identifying ploidy at the fry stage enabled ploidy to be determined in fish that may have otherwise died by the age when enough blood could be drawn for ploidy analysis. In addition, this technique made for a more efficient use of space since fish would not have to be reared to a size where blood samples could be drawn. To further advance ploidy detection techniques, ploidy may also be detected in fry before swim up, by microdissection to remove the yolk sac (Dr. Louis King, personal communication).

Silver Staining

Silver staining nucleoli has also been used to identify ploidy level of fish cells. Phillips et al. (1986) counted silver stained nucleoli to differentiate among haploid, diploid, and triploid rainbow trout, chinook, and coho salmon. Silver staining proved to yield good correlations between ploidy level and nucleoli number when ploidy level was subsequently varified using flow cytometry. Results showed that haploid individuals had one nucleolus per cell, diploid individuals had one or two nucleoli per cell, and triploid individuals had 1, 2, or 3 nucleoli per cell. However, this method can only be applied to species that have only one chromosome with a nucleolar organizer region (NOR) per haploid genome. For example, some members of the genus Salvelinus (lake trout and brook trout) have multiple chromosome pairs with NORs (Phillips and Ihssen 1985). Considerable variation occurs between different individuals of the same species in the total number of chromosomes with NORs, so that nucleoli counts may not be a reliable means of determining ploidy levels in lake trout, brook trout or hybrids involving these species.

Electrophoresis

Horizontal gel electrophoresis is used to detect differences in ploidy level by observing differences in staining intensities of specific polymorphic proteins.

Electrophoresis has been used to determine gene dosage in polyploid organisms, such as, grass carp (Wiley and Wike 1990), soft shell clams (Allen et al. 1986), and the amazon molly (Balsano et al. 1972). The effectiveness of electrophoretic assay, is dependent on the level of genetic variability inherent in the organism; the presence of heterozygous phenotypes is crucial. Correspondingly, the degree of resolution attainable in any electrophoretic study is directly proportional to the level of polymorphism (Allen et al. 1986).

In studies which utilize electrophoresis to determine ploidy, one must be conscious of the possibility of silenced gene products. A silenced gene product refers to the presence of a gene complex that fails to yield a product, in this case, a protein. For instance, the goldfish, is karyotypically tetraploid (Woods and Buth 1984). However, electrophoretic studies on this species, revealed that 81 percent of its enzyme coding loci were completely silenced (Woods and Buth 1984). Although this level of gene silencing is among the highest reported among tetraploid cypriniform fishes (Woods and Buth 1984), it does suggest a possible concern in using electrophoresis for ploidy determination. Complete loci expression for individual enzymes could be examined through comparative analysis using flow cytometry or other excepted techniques for determining ploidy level. Electrophoresis may not be a suitable method

for ploidy detection of bluegill fry because they are extremely small and may therefore prevent the extraction of a sufficient tissue sample. Additionally, electrophoresis may not distinguish between mosaic, aneuploid, or euploid organisms. Mosaic organisms may or may not express certain gene products. Also, aneuploid individuals can only be identified if the chromosomal location of enzyme coding gene segments are known.

MATERIALS AND METHODS

Experimental Animals

All bluegill used in this study were collected either by hook and line or seine. Collection sites included Lake Lansing, Haslett, Michigan; Lake Ovid, Haslett, Michigan; and Park Lake, Haslett, Michigan. Upon capture, all fish were immediately examined for ripeness. Ripe males and females were placed in a 163 liter cooler filled with lake water. Males were considered ripe if milt flowed easily from the genital pore when gentle pressure was applied simultaneously to both sides of the abdomen. Females were considered ripe if gentle pressure applied simultaneously to both sides of the abdomen released eggs, clear and round in appearance (Banner and Hyatt 1975; Westmaas 1992). The ripe fish were transported to the Aquaculture Research Laboratory at Michigan State University.

Well water, heated to 24°C was added to the cooler at approximately 2 liters/minute for a minimum of 30 minutes to acclimate the bluegill to lab water temperature. After acclimation, bluegill were removed from the cooler, separated according to sex, and placed into 41-liter rectangular tanks. Tanks were supplied with 24°C lab water at 0.5 liters/minute.

Description of Equipment

Incubation Cups

Design of incubation chambers was similar to that described by Westmaas (1992). Two part incubation chambers were made from PVC pipe of 5.0 cm and 6.4 cm inner diameter and 5 cm and 3.5 cm length, respectively. Nitex screen (420 μm mesh) was siliconed to one end of each piece of pipe. The larger cup was placed over the smaller cup to form an adequate enclosure to retain eggs and newly hatched fry, while allowing for oxygenated water transfer (Figure 1).

Cold Shock Unit

Three 460 liter oval fiberglass tanks (Frigid Units, Inc.) were equipped with water chillers (Frigid Units, Inc.) attached to one end of the tanks and one aerator each. The tanks were filled with approximately 400 liters of water each. Temperature was maintained at ± 0.5°C from the desired temperature and monitored using a digital thermometer (Corning Model 135). Accuracy of the digital thermometer was periodically checked using a hand held mercury thermometer (VWR, ASTM -18/82 C, Accuracy ± 0.5°C).

After fertilization, eggs were transferred to incubation cups and placed into a Heath incubation unit. At the time of shock initiation the incubation cups containing the eggs were transferred to the bottom of the

Figure 1. Photograph of two part PVC bluegill incubation chambers, placed in heath incubator unit. (A = top chamber, B = bottom chamber, C = top chamber over bottom chamber).

460 liter tanks at the end opposite of the chillers. Cold water instantly entered the incubation cups and surrounded the eggs. While maintaining a well circulating environment, the chillers created very little disturbance to the incubation cups, therefore eliminating any possibility of loss of eggs (Figure 2).

Pressure Shock Unit

A pressure shock unit based on the design by Dasgupta (1962) was used in the experiments. A hydraulic press (F.C. Carver, Inc., New York) equipped with a pressure gauge provided the desired pressure. Decompression speed could be regulated by adjustment of a threaded hydraulic release valve.

The treatment vessel of the pressure unit consisted of a cylinder and piston. The stainless steel cylinder was 13.97 cm long with an outer diameter of 5.08 cm and inner diameter of 3.81 cm. A 10.8 cm x 1.27 cm stainless steel base was welded to the base of the cylinder for stability. The solid brass piston was 17.78 cm long. Compression was maintained by two rubber "o" rings around the base of the piston. A release valve was located at the top of the piston that allowed the pressure to be quickly lowered after treatments were completed (Figure 3).

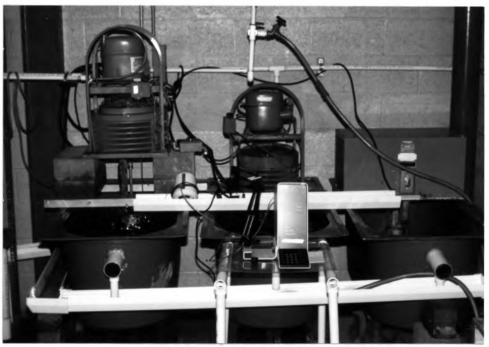

Figure 2. Photograph of cold shock unit used to treat fertilized bluegill eggs in the tetraploid induction experiments.

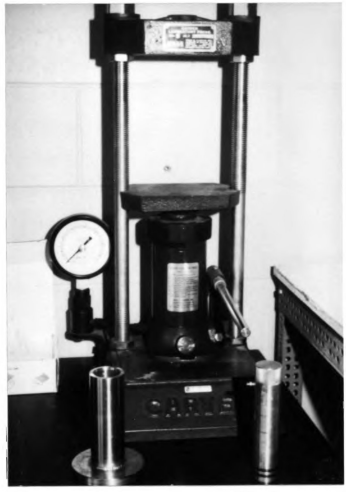

Figure 3. Photograph of pressure shock unit used to treat fertilized bluegill eggs in the tetraploid induction experiments.

Spawning Techniques

Bluegill were spawned using a hand spawning method described by Wilbert (1990). The genital area was dried and gentle pressure was applied to the sides of the female to expel eggs. A small initial sample of eggs was drawn from each female prior to use in the experiments. From 150 to 200 eggs were counted and weighed to the nearest 0.001 gram using a digital scale (Fisher Scientific Model 7303 DA). Based on these measurements, an approximate weight for subsamples containing between 1,000 to 1,500 eggs could be computed. Spawning of the female continued until all of the eggs were released. The eggs were randomly divided into subsamples of 1,000 to 1,500 eggs each. Each subsample was placed into an individual petri dish.

Milt was collected in a 14.6 cm pasteur pipet with bulb (WVR Scientific). The genital area was dried and gentle pressure was applied to both sides of the abdomen (Wilbert 1990). Milt from at least 6 males was combined and used to fertilize the subsamples of eggs obtained from one female. Equal amounts of milt were added to each subsample of eggs. If ripe males and females were captured in large numbers, eggs and sperm were randomly chosen from the captured population at the time of fertilization.

Immediately after milt was added to the eggs, eggs and milt were stirred gently to enhance fertilization (Wilbert 1990). For simplicity, this point was assumed to be the

time of fertilization, although actual fertilization probably occurred at some time shortly after the stirring process. A stopwatch (Micronta) was used to time the interval to shock initiation and shock duration. At 30 seconds, 24°C water was slowly added to the eggs to water activate the sperm and wash out excess milt (Piper et al. 1983; Wilbert, 1990).

The eggs obtained from each female provided two to three subsamples containing between 1,000 to 1,500 eggs each. Each set of subsamples from an individual female were randomly assigned, one as a control and the remaining as treatments. Subsamples from the same female were never subjected to the same treatment. Control samples were placed into incubator cups within one minute after water activation. The incubator cups were placed into the heath incubating unit supplied with 24°C water flowing at approximately 2.0 liters/minute.

Cold Shock Experiments

Fertilized eggs used for each cold shock treatment were placed into incubator cups within one minute after water activation. The incubator cups were placed into the heath incubating unit supplied with 24°C water flowing at approximately 2.0 liters/minute. The eggs were transferred to the cold shock unit at the time of treatment initiation.

A sample of 1,000 to 1,500 eggs was used for each cold shock treatment in an attempt to induce tetraploidy. Treatments were initiated at 35, 40, 45, and 50 minutes post fertilization. Temperatures and time of exposure ranged from 2.5°C to 10°C and 5 min to 20 minutes, respectively, and were based on the success of past research. We devised the connotation "thermal shock units" to describe the duration of the shock devided by the intensity. Past research (Westmaas 1992) was successful in producing two tetraploid bluegill using a shock of 5°C applied for 10 minutes, which would be equivalent to 2 "thermal shock units". Therefore, we designed a set of experiments based on 2 "thermal shock units". Three replicates along with controls were completed for each treatment (Table 4).

Pressure Shock Experiments

Fertilized eggs used in pressure shock treatments were placed into a pyrex dish (125mm x 65mm) supplied with 425 ml of 24°C water. The fertilized eggs remained in this dish until pressure shock treatment initiation at which time they were transferred to the pressure shock unit. Oxygen depletion was not a concern since a large amount of water was used relative to the amount of eggs. After completion of the pressure shock, the fertilized eggs were transferred to incubator cups and placed into a Heath incubating unit

Table 4. Initiation times, intensity, and duration of cold shock treatments applied to fertilized bluegill eggs. Fertilization is assumed to occur at the time in which the milt was added to the eggs.

Intensity (C)	Duration (min)	Initiation/Minutes Post Fertilization	Replicates
2.5	5	35	3
2.5	5	40	3
2.5	5	45	3
2.5	5	50	3
5.0	10	35	3
5.0	10	40	3
5.0	10	45	3
5.0	10	50	3
7.5	15	35	3
7.5	15	40	3
7.5	15	45	3
7.5	15	50	3
10.0	20	35	3
10.0	20	40	3
10.0	20	45	3
10.0	20	50	3

supplied with 24°C water flowing at approximately 2.0 liters/minute.

A subsample of 1,000 to 2,000 fertilized eggs from a female and approximately 15 ml of water were poured into the pressure cylinder just prior to applying the treatment. The piston was placed into the cylinder with its valve open. The piston was slowly pushed into the cylinder until water was observed exiting the valve. The valve was shut and the entire unit was placed under the press. Several preliminary pressure shock experiments were attempted during the spring of 1993. The treatments consisted of an intensity of 7,000 PSI applied for 10 minutes. Bluegill eggs were shocked at 20, 23, 25, 30, 32, and 35 minutes post fertilization. Additional pressure shock experiments, the results of which are reported in this manuscript, were initiated at 37, 40, 42, 45, and 50 minutes post fertilization. Intensities and durations of these shocks ranged from 6,000 to 8,000 PSI while durations ranged from 10 to 12 minutes (Table 5).

Incubation and Rearing

Eggs were incubated in a Heath incubation unit (Fig. 4). After hatching, which occurred 3 to 4 days post fertilization at 24°C, all dead or unhatched eggs were removed to prevent the growth of fungus which could contaminate the remaining live eggs. In many cases, eggs that did not hatch were clumped together and stuck to the

Table 5. Initiation times, intensity, and duration of pressure shock treatments applied to fertilized bluegill eggs.Fertilization is assumed to occur at the time in which the milt was added to the eggs.

Intensity (PSI)	Duration(min)	Initiation/Minutes Post Fertilization	Replicates
6000	12	45	2
6000	12	50	2
7000	10	37	2
7000	10	40	2
7000	10	42	3
7000	10	45	5
7000	10	50	3
8000	10	37	2
8000	10	45	2

Figure 4. Photograph of heath incubation unit used to incubate bluegill embryos and fry.

bottom of the incubation cups. Consequently, it was difficult to siphon the dead eggs out of the incubation cups. In these cases, it was easier to use a plastic water bottle with a stream spout to temporarily flush the hatched fry from the cups into a pyrex dish (125mm x 65mm) containing 425 mL of 24°C well water. The incubation cup containing the dead eggs was thoroughly cleaned, disinfected (Liqu-Nox ®), and rinsed before the hatched fry were returned to it. Disinfection helped prevent the fungus that formed on the dead eggs from infecting the surviving fry.

Because yolk sac material would result in great distortions in the flow cytometry readings, fish were tested once they reached swim up. Swim up refers to the life stage when the yolk sac has been absorbed and individuals begin exogenous feeding. Swim up occurred between 6 and 7 days post fertilization when eggs were incubated at 24°C.

Calculation of Survival after Cold and Pressure Treatment

Survival is a critical consideration in establishing protocols for ploidy induction. Treatments producing high tetraploid induction rates may not be effective if survival is extremely low. To avoid biases between the fertilization rates of individual females, survival was estimated for every treatment replicate and control.

Subsamples containing an estimated 1,000 to 1,500 eggs each, were used as a control and one or two treatment

replicates. Estimates were used since counting eggs would have been time consuming and egg viability may have been detrimentally affected if the eggs had been exposed to handling and air during a counting process. Survival was estimated on the day of ploidy analysis (between 7 and 12 days post fertilization). The number of fry were counted for each of the treatment replicates and controls that contained eggs from the same female. Relative survival was calculated as follows: (%treatment survival/%control survival) x 100.

Two subsamples of eggs from each female were used for the pressured shock experiments. These subsamples were randomly assigned, one as a control and the other as a treatment. Three subsamples were used for cold shock treatments, with two serving as treatments and one as a control. Subsamples from the same female were subjected to different cold shock treatments. In doing so, maternal affect on survival as well as tetraploid yield could be quantified.

One way analysis of variance was performed on treatment survival rates and tetraploid yields across initiation times, within treatments consisting of the same intensities and durations. Eggs of similar maternal descent were assigned to the same treatment group. Each test consisted of six treatment groups (females) and twelve observations. A total of eight separate tests were completed, four for the

analysis of survival rates and four for the analysis of tetraploid yields.

Tukey's studentized test was used to examine changes in relative survival between treatments. Comparisons of relative survival were made between intensities, lumped over initiation times and females and also between initiation times, within groups, consisting of the same intensity and duration, and lumped over females.

Ploidy Testing

Whole fry were tested for ploidy level between 7 and 12 days of age (shortly after yolk sac absorption). Five to ten fry from each shock treatment replicate were tested for ploidy. In some treatment replicates, fewer than five fry survived. All the fry from these replicates were tested by flow cytometry.

A Vantage dual laser cytofluorograph and the Vantage Cytoflourograph 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 tested on an argon-ion laser setting of 488nm with a 0.5 W output.

Pulse-height histograms were generated by a vantage computer with ACQ-CYTE software in conjunction with the cytofluorograph. The histograms were quantitative measurements based on the fluorescense intensity of the

total amount of double stranded nucleic acids per cell. The number of cells analyzed per sample ranged from 5,000 to 15,000. Propidium iodide was used as the cell probe and is specific for all double stranded nucleic acids. RNAase was added to the staining solution to eliminate the double straded nucleic acids of RNA from being stained. Slight variations in the histogram peak positions occurred between sampling periods as amplitude settings changed. Variation in histogram peak positions also occured during any one sampling period due to variations in the photoreceptors sensitivity. To insure proper identification of ploidy level, rainbow trout blood was used as an internal standard with every sample. Rainbow trout DNA is larger, generating histogram peaks to the right of diploid and tetraploid bluegill and therefore provided an additional peak in each sample and allowed for the calculation of a DNA index. A DNA index is necessary to quantify and justify ploidy level determination. A sample containing a known diploid bluegill and rainbow trout cells was used to establish DNA index values for diploid, triploid, and tetraploid bluegill. DNA index values were calculated by deviding the mean DNA fluorescense value of rainbow trout by the mean fluorescense value of the bluegill sample. Using the known sample of diploid bluegill, the DNA index for diploid bluegill was determined to be 2.8. A triploid individual should have exactly 1.5 times the number of chromosomes and 1.5 times

the mean DNA fluorescense of a diploid. Therefore, the DNA index of a triploid bluegill was set at 1.8. Tetraploid bluegill should have a DNA index of 1.4, since their mean DNA fluorescense value is twice that of a diploid.

The preparation of samples and the composition of reagents used in flow cytometric determination of ploidy in bluegill were simplified from techniques used by Westmaas (1992). The improved sampling procedures and protocols used for testing bluegill fry ploidy level using flow cytometry were as follows:

1. Seven to twelve day old swim up fry were placed (1 each) into 12 x 75 mm plastic test tubes that contained three drops of citrate buffer solution dispensed from a 14.6 cm pasteur pipet.

2. Fry were crushed using a glass stirring rod until no large particles were visible.

3. Citrate buffer solution (0.5 mL) was added to each sample tube. Each sample was triturated several times using a syringe equipped with a 25 gauge needle.

4a. One drop of rainbow trout blood[1] solution was added to each sample for use as an internal standard.

4b. Rainbow trout blood solution was prepared by adding 2 drops of rainbow trout blood to 2 mL of Citrate buffer solution in a test tube and then mixing.

5. Samples were centrifuged for 5 minutes at 1200 rpm.

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[1] Chinook salmon 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 rainbow trout blood if it is not readily available.

6. Supernant was removed from each of the samples by quickly inverting the test tubes.

7. Staining solution (1.0 mL) was added to each sample.

8. Samples were vortexed to resuspend cells.

9. Samples were analyzed on the Vantage Cytofluorograph.

The reagents required to make 100 mL of staining solution were as follows:

1. (0.1 ml) of (0.1 M) EDTA

2. 1.0 ml Triton X "10%".

3. 5.0 mg RNAse A in 1.0 ml H_20.

4. 5.0 mg Propidium Iodide in 5.0 ml phosphate buffer solution.

5. 93.0 ml Phosphate Buffer Solution (pH = 7.4).

RESULTS

Ploidy levels of bluegill fry were examined by flow cytometry. Diploid bluegill generated histogram peaks of approximately 35 units of DNA fluorescence for the G1 cell phase and a mean histogram peak value of 70.1 for the G2 cell phase (Figure 5). The DNA index was calculated by deviding the mean DNA fluorescense value of rainbow trout by the bluegill sample mean DNA fluorescense value. Diploid bluegill generated a DNA index of 2.8 (standard deviation = 0.03). Tetraploid bluegill peaks ran at 2 times the diploid peak or 450 to 600 units (Figure 6). Correspondingly, tetraploid bluegill generated a DNA index of 1.4 (standard deviation = 0.04). Triploid bluegill peaks ran at 1.5 times the diploid peak (Figure 7). Triploid bluegill generated a DNA index of 1.8 (standard deviation = 0.02).

Throughout the two years of this study a total of 25 separate shock treatments were examined. Cold shocks comprised 16 of these treatments. Three replicates, each with a control, were completed for all of the 16 cold shock treatments examined. Three of the 16 cold shock treatments produced tetraploids (Tables 6 and 7).

Three subsamples of eggs were used from each female for the cold shock experiments. Two of the subsamples were treated with cold shocks of the same intensity and duration,

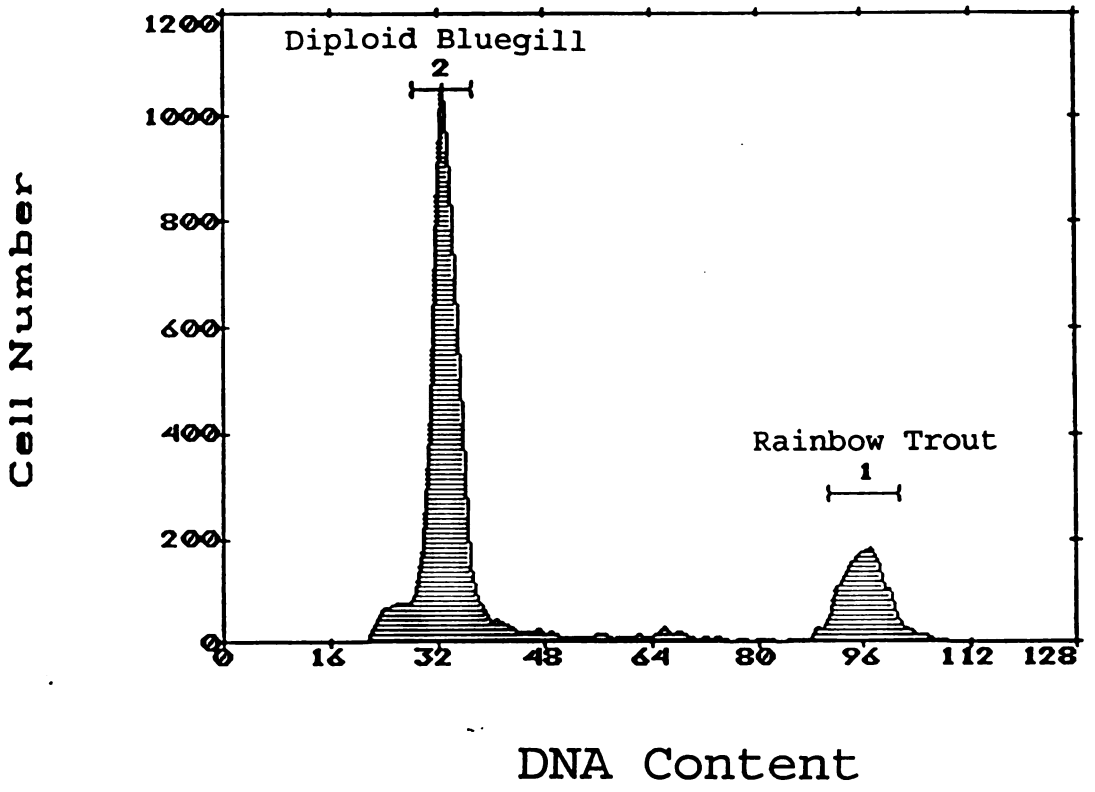

Figure 5. Example histogram peaks showing units of DNA fluorescence for diploid bluegill sunfish and rainbow trout cells, generated by flow cytometer. Diploid bluegill with G1 cell phase mean fluorescense of 33.1 and DNA index of 2.9.

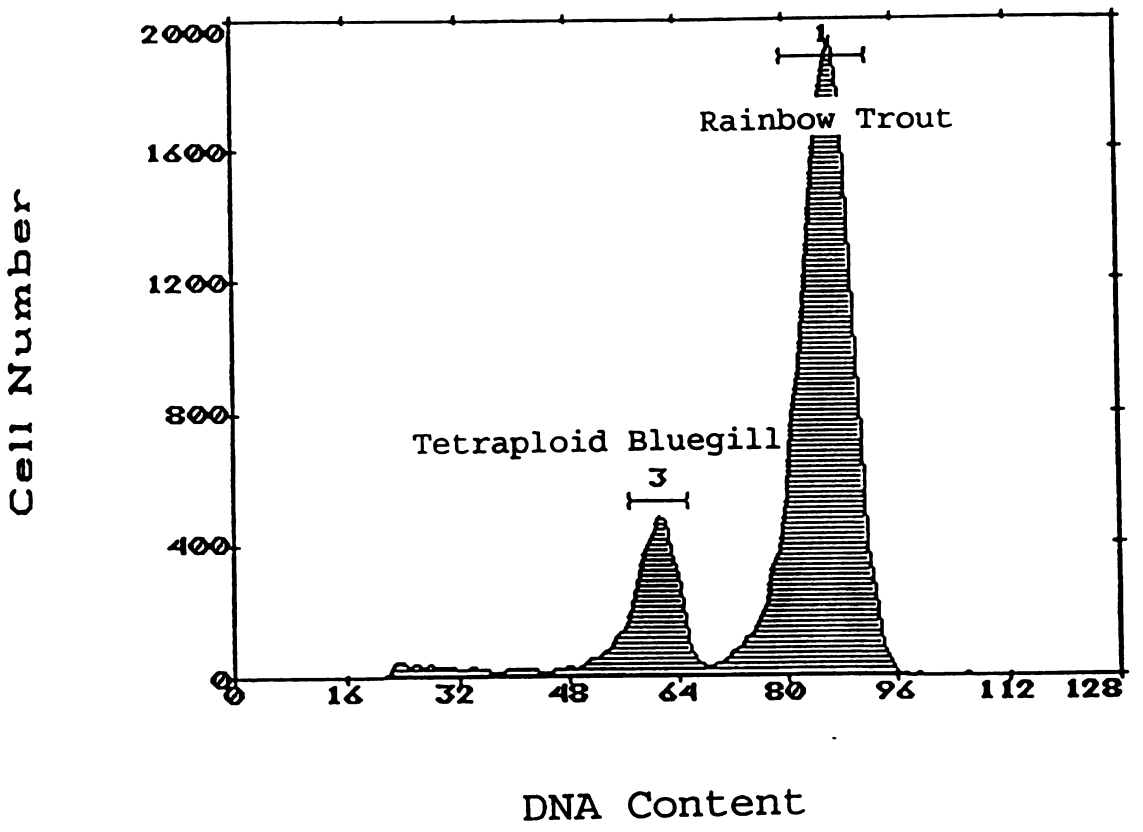

Figure 6. Example histogram peaks showing units of DNA fluorescence for tetraploid bluegill and rainbow trout cells generated by flow cytometer. Tetraploid bluegill with G1 cell phase mean luourescense of 61.0 and DNA index of 1.4.

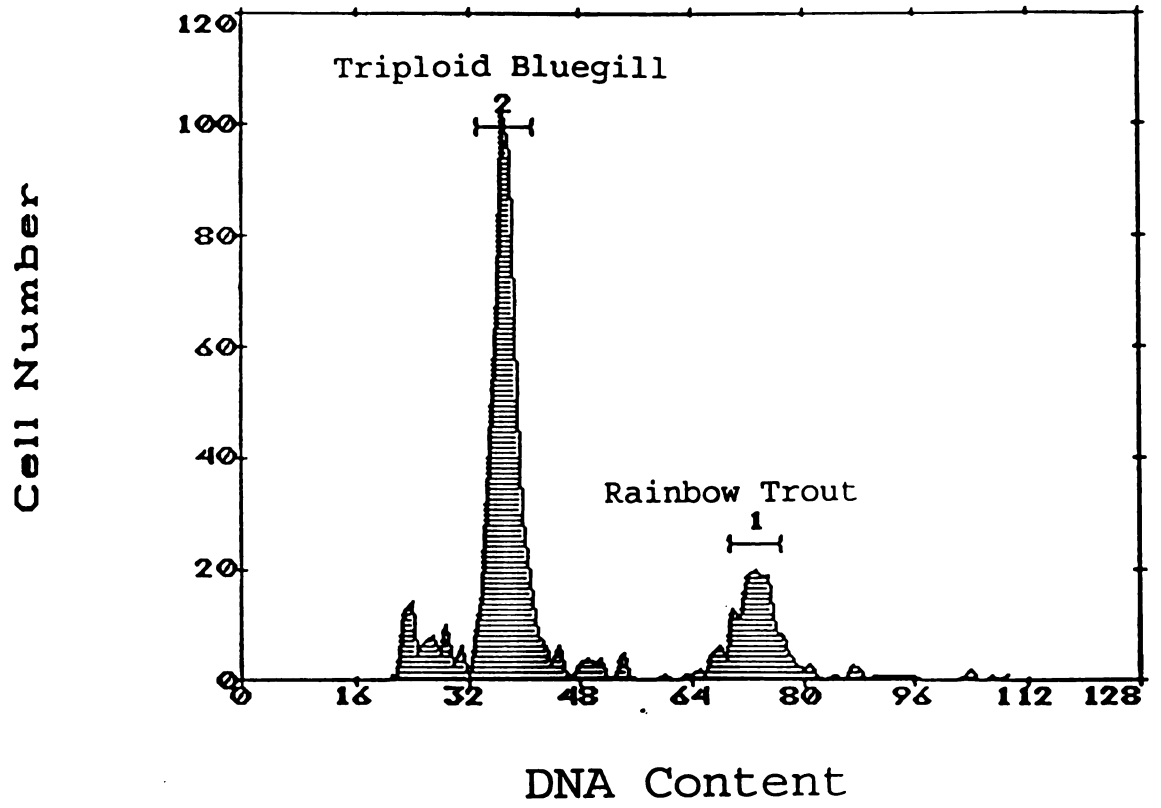

Figure 7. Example histogram peaks showing units of DNA fluorescence for triploid bluegill and rainbow trout cells generated by flow cytometer. Triploid bluegill with G1 cell phase mean fluourescense of 37.3 and DNA index of 1.82.

Table 6. Sample yield and total yield of tetraploidy in 7 to 12 day old bluegill fry from tetraploid induction experiments using cold shock treatments of 2.5 C applied for 5 minutes and 5.0 C applied for 10 minutes, initiated at 35 to 50 minutes post fertilization.

Intensity (C)	Duration (min)	Initiation (min)	Number Tested	Sample Yield[1]	Total Yield[2]
2.5	5	35	0		
2.5	5	35	5	0:5	0:50
2.5	5	35	0		
2.5	5	40	0		
2.5	5	40	4	0:4	0:4
2.5	5	40	0		
2.5	5	45	5		
2.5	5	45	3	0:10	0:17
2.5	5	45	2		
2.5	5	50	5		
2.5	5	50	1	0:6	0:11
2.5	5	50	0		
5.0	10	35	0		
5.0	10	35	8	0:13	0:64
5.0	10	35	5		
5.0	10	40	3		
5.0	10	40	5	0:10	0:10
5.0	10	40	2		
5.0	10	45	10		
5.0	10	45	5	0:19	0:52
5.0	10	45	4		
5.0	10	50	10		
5.0	10	50	5	0:16	0:56
5.0	10	50	1		

[1] Sample yield refers to the number of tetraploid fry out of the number of fry tested.

[2] Total yield refers to the number of tetraploid fry out of the total number that survived the treatment (%Sample yield x Number surviving treatment).

Table 7. Sample yield and total yield of tetraploidy in 7 to 12 day old bluegill fry from tetraploid induction experiments using cold shock treatments of 7.5 C applied for 15 minutes and 10.0 C applied for 20 minutes, initiated at 35 to 50 minutes post fertilization.

Intensity (C)	Duration (min)	Initiation (min)	Number Tested	Sample Yield[1]	Total Yield[2]
7.5	15	35	5		
7.5	15	35	1	0:10	0:19
7.5	15	35	4		
7.5	15	40	2		
7.5	15	40	2	2:5	2:5
7.5	15	40	1		
7.5	15	45	1		
7.5	15	45	1	1:13	1:13
7.5	15	45	11		
7.5	15	50	5		
7.5	15	50	0	0:11	0:11
7.5	15	50	6		
10.0	20	35	1		
10.0	20	35	10	0:16	0:73
10.0	20	35	5		
10.0	20	40	4		
10.0	20	40	10	1:19	1.6:30
10.0	20	40	5		
10.0	20	45	3		
10.0	20	45	1	0:9	0:69
10.0	20	45	5		
10.0	20	50	10		
10.0	20	50	0	0:15	0:100
10.0	20	50	5		

[1] Sample yield refers to the number of tetraploid fry out of the number of fry tested.

[2] Total yield refers to the number of tetraploid fry out of the total number that survived the treatment (%Sample yield x Number surviving treatment).

but different initiation times. The remaining subsample served as a control. Analysis of variance was used to evaluate the effect of maternal descent on tetraploid yield. One way analysis of variance was performed on tetraploid yield over the various initiation times, within treatments consisting of the same intensities and durations. Eggs of similar maternal descent were assigned to the same treatment group. No significant ($p < 0.49$) maternal effect was revealed at any of the four intensity levels (2.5, 5.0, 7.5, or 10.0°C).

Total yield is displayed as the total number of tetraploids produced by a treatment out of the total number of fry that survived that treatment to the testing date. An estimate for the total number of tetraploids produced by a treatment was calculated by multiplying the percentage of tetraploids among the tested subsample from a paticular treatment by the total number of fry surviving that paticular treatment. Treatments utilizing a 15 minute exposure to 7.5°C water, initiated at 40 minutes post fertilization, yielded a total of 2 tetraploids out of 5 fish that survived to swim up. A similar treatment utilizing a 15 minute exposure to 7.5°C water, initiated at 45 minutes post fertilization produced a total of one tetraploid out of 13 fish that survived the treatment. Treatments using 10°C water, 20 minute exposure, initiated at 40 minutes post fertilization, produced 1 tetraploid out

of 19 fish examined. Total yield from this treatment was 1.6:30.

Pressure shocks were initiated between 37 to 50 minutes post fertilization. A total of 9 different pressure shock treatments were examined. At least 2 replicates were performed for each of the nine pressure shock treatments examined. No tetraploid bluegill fry were detected from any of the pressure shock treatments (Table 8).

Each replicate of all the cold shock treatments and 4 of the 9 pressure shock treatments contained a control. Survival of controls ranged from 45:1000 to 565:1000 and averaged 262:1000 (Tables 9, 10, and 11). Average relative survival of pressure shock treatments was not significantly different than average relative survival of cold shock treatments ($p<0.50$).

Analysis of variance was used to evaluate maternal effect on egg survival. One way analysis of variance was performed on treatment survival rates over the various initiation times, within treatments consisting of the same intensities and durations. Eggs of similar maternal descent were assigned to the same treatment group. Of the four tests performed, one at each intensity level (2.5, 5.0, 7.5, and 10.0°C), only one revealed a significant result ($p < 0.02$). A significant maternal effect was revealed among the treatments subjected to a 10.0°C intensity. However, upon further examination, it was revealed that an unusually high

Table 8. Sample yield and total yield of tetraploidy in 7 to 12 day old bluegill fry from polyploidy induction experiments using pressure shocks (6000 PSI for 12 minutes), (7000 PSI for 10 minutes), and (8000 PSI for 10 minutes) initiated at 37 to 50 minutes post fertilization ("-" data missing).

Intensity (PSI)	Duration (min)	Initiation (min)	Number Tested	Sample Yield	Total Yield
6000	12	45	5	0:11	0:41
6000	12	45	6		
6000	12	50	1	0:6	0:38
6000	12	50	5		
7000	10	37	0	0:0	0:0
7000	10	37	0		
7000	10	40	0	0:5	0:-
7000	10	40	5		
7000	10	42	0		
7000	10	42	0	0:1	0:1
7000	10	42	1		
7000	10	45	0		
7000	10	45	0		
7000	10	45	3	0:3	0:3
7000	10	45	0		
7000	10	45	0		
7000	10	50	5		
7000	10	50	5	0:15	0:42
7000	10	50	5		
8000	10	37	0	0:0	0:0
8000	10	37	0		
8000	10	45	0	0:0	0:0
8000	10	45	0		

[1] Sample yield refers to the number of tetraploid fry out of the number of fry tested.

[2] Total yield refers to the number of tetraploid fry out of the total number that survived the treatment (%Sample yield x Number surviving treatment).

Table 9. Survival of 7 to 12 day old bluegill fry from tetraploid induction experiments using cold shock treatments of 2.5 C applied for 5 minutes and 5.0 C applied for 10 minutes, initiated at 35 to 50 minutes post fertilization.

Intensity (C)	Duration (min)	Initiation (min)	T_o[1]	C_o[2]	Rs[3]	Average Rs
2.5	5	35	0:1000	214:1000	0.00	
2.5	5	35	50:1000	124:1000	40.32	13.44
2.5	5	35	0:1000	237:1000	0.00	
2.5	5	40	0:1000	214:1000	0.00	
2.5	5	40	4:1000	124:1000	3.23	1.08
2.5	5	40	0:1000	237:1000	0.00	
2.5	5	45	12:1000	260:1000	4.62	
2.5	5	45	3:1000	321:1000	0.94	1.99
2.5	5	45	2:1000	504:1000	0.40	
2.5	5	50	10:1000	260:1000	3.85	
2.5	5	50	1:1000	321:1000	0.31	1.39
2.5	5	50	0:1000	504:1000	0.00	
5.0	10	35	0:1100	163:1100	0.00	
5.0	10	35	8:1200	176:1200	4.57	5.21
5.0	10	35	56:1000	506:1000	11.07	
5.0	10	40	3:1100	163:1100	1.82	
5.0	10	40	5:1200	176:1200	2.86	1.69
5.0	10	40	2:1000	506:1000	0.39	
5.0	10	45	21:1000	189:1000	11.11	
5.0	10	45	27:1000	321:1000	8.41	6.77
5.0	10	45	4:1000	497:1000	0.80	
5.0	10	50	50:1000	189:1000	26.45	
5.0	10	50	5:1000	321:1000	1.56	9.40
5.0	10	50	1:1000	497:1000	0.20	

[1] T_o = Treatment survival
[2] C_o = Control survival
[3] Rs = Relative survival
= (survival of shock treated fish/survival of control fish) X 100

Table 10. Survival of 7 to 12 day old bluegill fry from tetraploid induction experiments using cold shock treatments of 7.5 C applied for 15 minutes and 10.0 C applied for 20 minutes, initiated at 35 to 50 minutes post fertilization.

Intensity (C)	Duration (min)	Initiation (min)	T_o[1]	C_o[2]	Rs[3]	Average Rs
7.5	15	35	14:1000	273:1000	5.13	
7.5	15	35	1:1000	565:1000	0.18	2.65
7.5	15	35	4:1000	151:1000	2.65	
7.5	15	40	2:1000	273:1000	0.73	
7.5	15	40	2:1000	565:1000	0.35	0.58
7.5	15	40	1:1000	151:1000	0.66	
7.5	15	45	1:1000	471:1000	0.21	
7.5	15	45	1:1000	227:1000	0.44	2.24
7.5	15	45	11:1000	181:1000	6.08	
7.5	15	50	5:1000	471:1000	1.06	
7.5	15	50	0:1000	227:1000	0.00	1.46
7.5	15	50	6:1000	181:1000	3.32	
10.0	20	35	1:1000	176:1000	0.57	
10.0	20	35	32:1000	275:1000	11.64	33.70
10.0	20	35	40:1000	45:1000	88.89	
10.0	20	40	4:1000	176:1000	2.27	
10.0	20	40	21:1000	275:1000	7.64	7.01
10.0	20	40	5:1000	45:1000	11.11	
10.0	20	45	3:1000	317:1000	0.95	
10.0	20	45	1:1000	241:1000	0.42	6.77
10.0	20	45	65:1000	246:1000	26.42	
10.0	20	50	22:1000	317:1000	6.94	
10.0	20	50	0:1000	241:1000	0.00	12.88
10.0	20	50	78:1000	246:1000	31.71	

[1] T_o = Treatment survival
[2] C_o = Control survival
[3] Rs = Relative survival
= (survival of shock treated fish/survival of control fish) X 100

Table 11. Survival of 7 to 12 day old bluegill fry from tetraploid induction experiments using pressure shock treatments of 6000 PSI applied for 12 minutes, 7000 PSI applied for 10 minutes, and 8000 PSI applied for 10 minutes, initiated at 37 to 50 minutes post fertilization ("-" indicates data unavailable).

Intensity (PSI)	Duration (min)	Initiation (min)	T_o[1]	C_o[2]	Rs[3]	Average Rs
6000	12	45	35:1000	323:1000	10.84	
6000	12	45	6:1000	112:1000	5.36	8.10
6000	12	50	1:1000	391:1000	0.26	
6000	12	50	37:1000	64:1000	57.81	29.04
7000	10	37	0:-	-	0.00	
7000	10	37	0:-	-	0.00	0.00
7000	10	40	0:-	-	0.00	
7000	10	40	5:-	-	-	-
7000	10	42	0:-	-	0.00	
7000	10	42	0:-	-	0.00	-
7000	10	42	1:-	-	-	
7000	10	45	0:1000	181:1000	0.00	
7000	10	45	0:1000	276:1000	0.00	0.14
7000	10	45	3:1000	432:1000	0.69	
7000	10	45	0:-	-	0.00	
7000	10	45	0:-	-	0.00	
7000	10	45	10:1000	122:1000	8.20	
7000	10	45	27:1000	280:1000	9.64	6.43
7000	10	45	5:1000	348:1000	1.44	
8000	10	37	0:-	-	0.00	
8000	10	37	0:-	-	0.00	0.00
8000	10	45	0:-	-	0.00	
8000	10	45	0:-	-	0.00	0.00

[1] T_o = Treatment survival
[2] C_o = Control survival
[3] Rs = Relative survival
= (survival of shock treated fish/survival of control fish) X 100

survival rate obtained by the two subsamples of eggs from one female were responsible for the significant result. Reanalysis of this group of treatments after the removal of the suspected outlier values, resulted in a much lower significance value ($p < 0.19$). For the purposes of this research, where females were selected solely on the basis of egg ripeness, it can be argued that female effect on survival was negligable.

Relative survival of the cold shock treatments ranged from 0.58% to 33.70% (Tables 9 and 10). All 16 cold shock treatments produced fry that hatched and survived to be tested.

One way analysis of variance was used to examine the effects of treatment initiation times and intensities on survival. The time of treatment initiation did not have a significant effect on relative survival rate ($p < 0.72$). However, treatment intensity did cause a significant effect on relative survival rate. Tukey's (HSD) test further revealed that fertilized eggs subjected to a treatment intensity of 10.0°C had a significantly ($\alpha = 0.06$) higher survival rate than fertilized eggs subjected to a treatment intensity of 7.5°C (Table 12).

Relative survival of the pressure shock treatments ranged from 0.00% to 29.04% (Table 11). Ten of the 23 pressure shock replicates had fry that hatched and survived to be tested.

Table 12. Results of Tukey's (HSD) test of pair-wise comparisons of relative survival rates between cold shock treatment intensities, lumped over duration times, females, and initiation times. (Means not significantly different (α=0.06) carry the same superscript).

Intensity (C)	N	Mean	Standard Error
10.0	12	3.196[a]	0.738
5.0	12	2.138[ab]	0.393
2.5	12	1.604[ab]	0.466
7.5	12	1.364[b]	0.184

Nonparametric analysis of variance was used to evaluate the effect of pressure shock intensity on relative survival rates. The Kruskal-Wallis test of ranked data revealed no significant effect ($p < 0.18$) of pressure shock intensity on relative survival rates.

During the course of this study, approximately 15 to 20 fish (2.9% of the total that survived treatments) were observed to have marked malformations. These malformations were present only in the individuals subjected to cold shocks. The malformations included cases of what appeared to be scoliosis, lordosis, and abnormal enlargement of the head region. All of the affected individuals died before flow cytometry ploidy analysis could be performed.

DISCUSSION

Cold and pressure shock treatments were administered between 35 and 50 minutes post fertilization. Tetraploid bluegill were produced with cold shocks applied at 40 and 45 minutes post fertilization but not at 35 or 50 minutes post fertilization. Tetraploidy was induced in 40.0% of the fry tested after application of a cold shock treatment of 7.5°C applied for 15 minutes at 40 minutes post fertilization. Treatments utilizing a 15 minute exposure to 7.5°C water, initiated at 45 minutes post fertilization induced tetraploidy in 7.7% of the fry tested. Treatments using 10.0°C water, 20 minute exposure, initiated at 40 minutes post fertilization resulted in a 5.3% tetraploid induction rate. Westmaas (1992) administered cold and pressure shocks to bluegill eggs at 20 to 45 minutes post fertilization. Westmaas (1992) reported 5 and 10 percent tetraploid induction using cold shock treatments of 5°C, applied for 10 minutes at 40 and 45 minutes post fertilization respectively. Shocks applied before 40 minutes resulted in 0% tetraploid induction (Westmaas 1992). The results reported by Westmaas (1992) were consistent with ours.

This seems to contradict the notion that tetraploidy is

induced just prior to and during the first mitotic division of the fertilized egg (Thorgaard 1986; Komen et al. 1991), which in bluegill occurs on average at 35 minutes post fertilization when incubated at 22°C (Morgan 1951; Montes-Brunner 1992). In our experiments, pre-shock acclimation temperatures were maintained at 24 ± 1°C. Although by most indicators, increased water temperature appears to increase the rate of egg development, it is possible the elevated temperatures the bluegill were acclimated in may have retarded the rate of egg development as well as the time to the first mitotic division.

Laboratory water temperature was very similar to lake temperatures at the time and place of collection. Lake water temperature during collection ranged from 22°C to 28°C. However, spawning activity was frequently interrupted due to changes in lake water conditions such as increased wave activity or decreased temperature. Spawning activity slowed considerably, if not totally, during cold periods or heavy rains. Without constant conditions, specifically temperature, it was likely that rate of egg development also fluctuated during the spawning season. Consequently, upon entering the lab, degree of egg development may have varied between females.

Montes-Brunner (1992) reported that the first mitotic division of the fertilized bluegill egg occurred on average at 35 minutes post fertilization at 22°C. If eggs exhibited

a slower rate of development than reported by Montes-Brunner (1992), it is probable that they were shocked prior to first cleavage at 40 to 50 minutes post fertilization thus producing tetraploids. If the rate of egg development was similar to the rate of egg development reported by Montes-Brunner (1992) then the shocks initiated at 40 to 50 minutes post fertilization produced tetraploids by inhibiting the second mitotic division which occured at 55 minutes post fertilization (Meyer 1951; Montes-Brunner 1992). The second mitotic division refers to the process of forming 4 blastomeres from the 2 blastomere stage. Tetraploid formation at this point would involve the inhibition of karyokinesis or cytokinesis in both of the 2 blastomeres. If the second mitotic division was suppressed in only one of the two blastomeres, both tetraploid and diploid cells would continue to be produced. This condition is referred to as mosaic. Past research has indicated the production of mosaics (Diter et al. 1993) and aneuploids (Chourrout 1986) when the second mitotic division was partially interrupted. If mosaic bluegill were produced and survived to be tested, they would have appeared on the flow cytometer as a mix of both tetraploid and diploid cells. Flow cytometry revealed no mosaic bluegill fry during the course of this experiment.

Cold Shocks

The cold shock units developed for this study were extremely efficient and easy to use. The duration of the cold shock treatments ranged from 5 to 20 minutes of exposure. Therefore, an essential component of the cold shock treatments was the ability to regulate and maintain desired temperatures. Temperature could be adjusted and maintained at the desired temperature ± 0.5°C. The chillers also provided circulation and aeration.

Cold shock treatments were the only method that produced tetraploids. Tetraploidy was induced at a maximum rate of 40% when eggs were exposed to 7.5°C water for 15 minutes at 40 minutes post-fertilization. However, total tetraploid yield was only 2:5. On three attempts, this treatment produced only 5 fish that survived to be tested. Although total yield of tetraploids is low, the number of eggs subjected to this treatment could be substantially increased. Therefore, the commercial feasability of using this protocol for producing tetraploid bluegill can be substantiated. For example, adult female bluegill produce between 6,000 to 9,000 eggs in any one spawning event (Garling, personal communication). Assuming similar survival rates and tetraploid yields to those obtained with the above treatment, and using 2 males to fertilize the eggs from each female, 480 ripe adults, consisting of 160 females and 320 males could produce a total of 2,784 tetraploids

surviving to swim up. These individuals could be used to establish a tetraploid broodstock, which in turn could be used to produce triploids. However, several factors including genetic concerns must be considered before initiating a broodstock. Appendix B describes these factors in greater detail.

The results from this experiment suggest that lower temperature shocks (7.5°C and 10.0°C) improved the production of tetraploids. Westmaas (1992) produced tetraploid bluegill using cold shocks of 5.0°C applied for 10 minutes. Westmaas (1992) also suggested that the treatment may have been too harsh in intensity, duration, or both. Although induction rates were improved from the work of Westmaas (1992) relative survival rates of cold shock treatments remained very low (< 33.07%).

The highest rate of tetraploid induction was achieved with a shock of 7.5°C applied for 15 minutes at 40 minutes post fertilization. Interestingly, the 7.5°C intensity level had the lowest relative survival rates. Diter et al. (1993) suggested that while percent tetraploid production could be increased by increasing the shock intensity, survival rate would subsequently decrease. Cold shock treatments of 2.5°C and 5.0°C intensity, failed to produce tetraploids. Tetraploidy induction was improved with treatments of 7.5°C and 10.0°C. Tetraploids were therefore produced using treatments of lower temperatures intensities.

However, although the temperature of the shocks was lower, the durations were higher. Further refinements in the duration of the cold shock treatments may improve both survival and tetraploid induction.

The highest induction rate (40.0%) attained in this study is comparable to induction rates produced in other fishes. Thorgaard et al. (1981) reported 16 percent tetraploidy induction in rainbow trout using a heat shock initiated at 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 and 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 (1988) reported 25 percent tetraploidy induction in tilapia using cold shocks.

Pressure Shocks

Pressure shocks were initiated between 37 and 50 minutes post fertilization and ranged from 10 to 12 minutes in duration. Shock intensities of 6,000, 7,000, and 8,000 PSI were used. No tetraploids were produced from a total of 9 different pressure shock treatments examined. Westmaas (1992) administered pressure shock treatments of 8,000 PSI

applied for 5 minutes in an attempt to induce tetraploidy in bluegill. Westmaas (1992) reported 0% tetraploid induction from shocks applied at 20, 25, 30, 35, and 40 minutes post fertilization. Although Westmaas (1992) did not have success inducing tetraploidy with pressure shocks, pressure was used to induce 100% triploidy. Based on the findings of Thorgaard et al. (1981), who used the same shocks applied at different initial times to induce both triploidy and tetraploidy in rainbow trout and also because of the limited number of experiments using pressure to induce tetraploidy in bluegill (Westmaas 1992), it was felt that further investigation on pressure shocks was warranted.

Westmaas (1992) reported 0% tetraploidy induction in bluegill eggs subjected to 8,000 psi for 5 minutes. We used pressure shocks of lower intensities and longer durations than Westmaas (1992). It is possible that different intensities, durations, and initiation times could be used to induce tetraploidy in bluegill. Diter et al. (1993) suggested that the efficient application time of temperature shocks to produce tetraploids is much broader than that of pressure shocks. In addition, they suggested that heat shocks actually interfere with factors involved in the migration of whole chromosome sets (such as aster formation), while pressure treatments may impair the processes involved in the migration of individual chromosomes (such as microtubules of the mitotic spindle).

In such a case, one would expect the possible formation of aneuploid cells when pressure shocks are applied. Aneuploidy refers to the presence of unequal numbers of individual chromosomes within a cell, caused by either an addition or deletion of chromosomes. Preliminary pressure shock experiments may have produced aneuploid bluegill. Flow cytometry analysis revealed four individuals that had DNA measurements 1.5 times the size of diploid controls. Three of these individuals were exposed to 7,000 PSI for 10 minutes, initiated at 23 minutes post fertilization, while the fourth individual was exposed to 7,000 PSI for 10 minutes, initiated at 40 minutes post fertilization. The DNA measurements resemble those of triploid bluegill, however the treatments were initiated several minutes after the second meoitic division. In this case flow cytometry could not positively identify the cells as triploid or aneuploid. Aneuploidy is often a lethal condition (Griffiths et al. 1993), however, aneuploid rainbow trout have been identified through karyotypes (Chourrout 1986). It is unknown whether aneuploids were produced in this experiment. If aneuploids were produced, the condition may have been fatal in some cases, while not in others.

Survival was recorded between 7 and 12 days post-fertilization. Both treatments and controls exhibited low survival. Average survival of controls between the ages of 7 to 12 days was 26.2%. Using similar spawning and

incubation methods, Westmaas (1992) reported an average survival of 83.06% for three control replicates to five days post fertilization. Although Westmaas (1992) reported higher survival rates, the females used for this analysis were collected on the same day, therefore, these results may not reflect the survival of eggs collected throughout the entire spawning season. Additionally, Westmaas (1992) reported survival rates at 2 days post hatching. At this time bluegill obtain all of there nutritional requirements from the yolk sac. Bluegill begin exogenous feeding at 6 to 7 days post fertilization, or 3 to 4 days post hatching when incubated at 24°C (Wilbert 1990). Survival rates in this experiment were estimated at 7 to 12 days post fertilization or 5 to 10 days post hatching. Because bluegill fry are very small, it was necessary to hold them in the incubation cups until they were removed for analysis. The incubation cups were sealed at both ends with very small mesh screen (420 μm mesh) and at times, several trays of the Heath incubation unit were stacked full of the cups. The cups, nor the incubation unit provided the necessary circulation for effluent and waste discharge. Therefore, the bluegill fry were not fed during this experiment. Consequently, it is possible that mortalities could have occurred due to starvation before survival was estimated. However, the majority of the mortalities in both the treatments and the controls occurred during the initial stages of embryo

development, prior to hatching. Wills et al. (1994) reported an average control survival of 55.0 ± 31.3% for female bluegill x green sunfish hybrids, which compares more favorably with the results of our work.

Low survival of the controls in our research may be attributed to the fact that the eggs from each female were stripped entirely. Perhaps only a portion of the eggs within a female were in a optimally ripe condition. If all of the eggs were not in a optimally ripe condition, then one would expect that the suboptimal eggs would not survive as well as the others. Additionally, suboptimal eggs would withstand a better chance of surviving control conditions as opposed to being subjected to a treatment. Therefore, the results of this experiment may, inpart, reflect a bias in survival rates because of egg condition. Westmaas (1992) also used three subsamples of eggs from each of three females to estimate survival of controls and treatments. However, Westmaas (1992) did not indicate the number of eggs used per subsample or how the eggs were chosen. The results of our experiment indicate that the eggs within a female may vary in their condition and the methods used to obtain these eggs may impact the results.

An additional source of mortality could have resulted from the timing of the shocks. Tetraploidy was induced by treatments that were initiated at 40, 45 and 50 minutes post fertilization. If the rate of embryo development among the

experimental units in this work, was similar to the rates of bluegill embryo development reported by Morgan (1951) and Montes-Brunner (1992), then one would expect that tetraploidy was induced by the inhibition of the second mitotic division. The production of mosaics has been reported when shocks were applied during the second mitotic division of rainbow trout embryos (Chourrout 1986; Diter et al. 1993). Mosaics have also been observed by Thorgaard et al. (1981), Chourrout (1982), and Bidwell et al. (1985), in tetraploidy inducing trials using heat shocks. Mosaic bluegill may have been produced in this experiment because of shock treatment initiation times. Mosaic cell configurations may be lethal to bluegill. If mosaics are lethal to bluegill, significant mortalitiy could have occured due to mosaic induction.

Average relative survival of eggs treated with cold shocks was 6.92 percent. Westmaas (1992) reported that survival rates of bluegill eggs subjected to cold shocks was low although no specific numbers were given. Induction of tetraploidy was higher than that reported by Westmaas (1992). The treatments that induced tetraploidy at the highest rates, consisted of lower temperatures (7.5°C and 10.0°C) than the 5.0°C intensity treatments used by Westmaas (1992). Although the intensity was lower, the time of exposure was longer than those attempted by Westmaas (1992). It is possible that a decrease in the time of exposure while

maintaining the same intensity could increase survival with out decreasing tetraploid yield.

Relative surival of eggs subjected to pressure shock treatments was also low, averagig 6.24 percent. According to Morgan (1951) and Montes-Brunner (1992) the first mitotic division of the fertilized egg occurs at 35 minutes post fertilization. If the first mitotic divison did occur at this time under our conditions, this may represent a more sensistive period of embryo development. Diter et al. (1993) suggested that pressure shocks may impair the processes involved in the migration of individual chromosomes, such as the formation of microtublules of the mitotic spindle. The pressure treatments applied during the first mitotic division may have caused the production of aneuploids, resulting in high mortalities.

Low survival was observed in many of the tetraploid replicates. The moderate to low induction rates and low survival of the shocked eggs suggests that refinements in the cold and pressure shocks durations and intensities should be made. In addition, survival may have been reduced due to escapement from the incubation units as well as starvation. However, the majority of the mortality in all the shock treatments occurred during the initial stages of embryo development, prior to hatching. Survival estimates may be higher if data were collected shortly after hatching. At this stage of development the fry have very little

mobility, therefore, escapement through the top of the incubation units is minimized, if not eliminated. In addition, at this early fry stage, there is little threat to starvation, because the fry utilize the yolk sac for their nutritional requirements. However, survival estimates taken shortly after hatching may not reflect true survival of treatments if the treatments or tetraploid induction creates mortality at later stages of development.

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 (Thorogaard et al. 1981); but was 40 percent for tetraploid rainbow trout produced by pressure shocks (Chourrout 1984).

The low survival of tetraploids may have been the 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.

Various cases of disembryogenesis were observed in the bluegill fry of this experiment. Disembryogenesis refers to the abnormal development of an embryo. Approximately 15 to

20 bluegill fry (approximately 3% of total that survived treatments) were observed to have marked malformations, including cases of scoliosis and abnormally enlarged head regions. All of these individuals died before flow cytometry analysis could be performed. In addition, all of the observed malformations occurred among fry subjected to cold shock treatments. Disembryogenesis was not observed among bluegill fry subjected to pressure shocks or controls.

The most crucial component to any chromosomal manipulation study is the availability of large quantities of viable, male and female gametes of the appropriate species. In this study, fish were captured from natural bodies of water by seine as well as hook and line. Capture sites close to the laboratory were necessary, so fish could be used before egg degeneration. Observations made during the first year of this project indicated that egg quality appeared to decline after approximately 24 hours of holding. Two disadvantages of capturing spawning bluegill from natural populations included the seasonally narrow window in which to capture the fish and the capture of unequal amounts of males and females. In addition, tremendous time was devoted to both capturing the fish and on preseason monitoring.

As mentioned previously, weather conditions fluctuated greatly during the spawning seasons in 1993 and 1994. Decreases in lake water temperature caused by decreases in

air temperature or rainfall slowed or completely stopped spawning activity. Perhaps the strongest influence on the results of this experiment were the fluctuations in the lake temperature created by variations in weather patterns. It is very likely that the temporary fluctuations in lake water temperature during the spawning season could cause variations in the rate of egg development. For instance, many females were captured and upon examination of ripeness, were pronounced almost ripe, with perhaps two days of further maturation required. A cold period in the weather would then virtually stop the spawning activity for as much as two weeks. Upon resuming spawning activity, these females that were almost ripe may now contain eggs that are in a slower rate of development because of the cold period. Consequently, the results of this experiment may, inpart, reflect the impact of fluctuating environmental conditions. If rate of egg development was not consistent between samples, then replications of the treatments did not occur. The central range of the bluegill may provide more stable climatic conditions. Similar experimentation in these areas may achieve more precise results. Perhaps the most ideal approach to experimentation of this sort would be to utilize brood fish from captive populations. If bluegill could be raised and induced to spawn in a controlled culture environment, variations in egg development rates could be reduced.

Methods to artificially induce spawning within the laboratory would greatly increase the efficiency of this research. Laboratory spawning could expand the spawning season to a 12 month cycle, providing ovulating adults throughout the year. In addition, laboratory spawning would eliminate fluctuations in climate conditions (temperature) and therefore reduce potential variation in rate of egg development. Using similar methods to Banner and Hyatt (1975), our efforts to induce out of season spawning in a laboratory failed (Appendix A). Bryan et al. (1994) successfully developed laboratory culture and artificial spawning techniques for bluegill that provided ovulating females and ripe males continuously throughout the year.

In addition to changing weather conditions, catches from natural populations are often biased towards one sex. Males are most frequently captured during early season spawning, while toward the end of the season, ovulating females were captured at higher frequencies than ripe males. Cryopreservation of sperm could help alleviate this problem. Storage of sperm would allow the fertilization of more females, thereby increasing the number of possible treatments. Cryopreservation techniques have been developed for several species of fish including channel catfish (Tiersch et al. 1994), rainbow trout (Holtz, 1993), walleye, muskellunge (Moore 1991), and yellow perch (Ciereszko et al. 1993). Although cryopreservation is a promising method to

increase the number of spawns, one question remains to be answered: What adverse chromosomal modifications may occur in the sperm exposed to cryopreservation? Cryopreservation techniques vary considerably between species and protocols for the cryopreservation of bluegill semen need to be established.

SUMMARY AND CONCLUSIONS

Summary

The goal of this research was to improve tetraploid induction protocols in bluegill sunfish in an effort to provide both fisheries managers and aquaculturists with tools to enhance bluegill management practices and culture techniques. Previously developed pressure shock treatments have yielded 100% triploids with high relative survival (Westmaas, 1992). However, the short window of egg availability, labor intensive collection and spawning techniques, and the expense of ploidy analysis, may negate the benefits of using pressure to induce triploidy directly.

This research was successful in developing techniques to produce tetraploid bluegill at a maximum yield of 7.7:69. Although relative survival of bluegill subjected to this treatment was low (9.26 percent), the number of eggs treated could be increased substantially. Using this protocol, large numbers of tetraploid fish could be produced in one season. If tetraploid bluegill stocks could be produced and raised to maturity, the tetraploid X diploid cross may be used to effectively and economically produce triploids in the future. Other researchers have shown that a tetraploid

x diploid cross will produce triploids with higher survival and better overall performance than triploids produced directly by shock treatments (Chourrout et al. 1986). If tetraploids can 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 with much less expenditure of time and money.

Using the protocols developed by Westmaas (1992) bluegill were tested for ploidy level at a very early age (7 to 12 days). It is not known if tetraploidy is lethal in bluegill sunfish at later stages of development. 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. However, if tetraploidy is lethal to bluegill at later stages of development, then the tetraploids produced by Wilbert (1990) 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.

Conclusions

The results of this experiment indicate that

tetraploidy induction in bluegill sunfish is possible. Tetraploidy in bluegill is induced most efficiently with cold shock treatments. Continued refinement of cold shock applications could improve tetraploid induction. Intensities of 7.5°C maintained for 10 and 20 minutes should be attempted at 37, 40, 42, and 45 minutes post fertilization. Comparisons of early development and survival between tetraploid and diploid bluegill, as well as gonadal development in adults should be examined. Research at Southern Illinois University is currently investigating growth rates and gonad development in triploid bluegill x green sunfish hybrids. Preliminary results indicate that triploids have simlar growth rates to diploids up to a size of 1/4 pound. Additionally, triploids have significantly reduced gonad development (Sheehan, personal comm.).

Attempts should be made to establish brood ponds of tetraploid bluegill. Tetraploid X tetraploid and tetraploid X diploid crosses should be attempted. Additionally, measures of fertilization rates and gamete quality should be made.

Moderate tetraploid induction rates were achieved in this study. The induction of tetraploidy coincided with low survival. However, because tetraploids are fertile, a brood stock could be established to raise the overall population size. Following the tetraploid induction protocols established in this research, one could establish a

tetraploid broodstock that could be used to produce triploids. Several factors including genetic concerns must be considered when addressing this question. Appendix B describes how these factors should be incorporated with the existing tetraploid bluegill induction protocols to produce an effective breeding number for a tetraploid broodstock.

APPENDIX A

Methods to artificially induce spawning within a laboratory would greatly increase the efficiency of this research. Laboratory spawning could expand the spawning season to a 12 month cycle, providing ovulating adults throughout the year. In the winters of 1992-93 and 1993-94 attempts were made to induce bluegills to spawn in the Upper River Lab at Michigan State University using modified techniques of Banner and Hyatt (1975).

Bluegills used for artificial spawning experiments were captured through the ice by hook and line from Lake Ovid, Ovid, Michigan. Bluegills were transported back to the laboratory in a 163 liter cooler. Bluegills were identified in the field and identifications were confirmed at the laboratory (Eddy and Underhill, 1978). The cooler was supplied with an aeration unit and bluegill were allowed to acclimate to 12°C (Approximately 12 hours).

In the winter of 1992-93, 30 bluegill (15 males and 15 females, between 4.5 and 6.5 inches in total length) were transferred from the cooler with water temperature at 12°C to a 1200 liter fiberglass tank maintained at a temperature of 12°C and flow rate of 3 liters per minute. Prior to the

addition of the bluegill, pea gravel was spread along the entire bottom of the tank. Fish were maintained at 12°C and a photoperiod of 8 hours light and 16 hours dark for a period of 1 month. During this initial 1 month period, bluegill were fed twice daily with commercial trout feed (Zeigler, 5/32 inch pellets). Bluegill began feeding aggressively after the second week in the lab.

After the first month simultaneous adjustments in water temperature and photoperiod were initiated. Water was slowly heated by 1c every 2 days until a final temperature of 25°C was attained (approximately 24 days). Photoperiod was increased by 1 hour of light per week to reach a final adjustment of 16 hours light and 8 hours dark. Once the light and photoperiod adjustments were completed, fish were checked once a week for ripeness. Females were considered ripe when the eggs flowed easily from the genital pore when gentle pressure was applied simultaneously to both sides of the abdomen. Males were considered ripe if milt flowed easily from the genital pore when similar pressure was applied to the abdomen.

Most of the 15 males achieved sexual ripeness during the first week after final temperature and photoperiod adjustments were completed. These males maintained this state for approximately one month. Three of the largest males constructed nests from the pea gravel and demonstrated nest guarding behaviors.

Weekly examinations continued for a period of one month. During this time no ripe females were observed. After this time, male mating behavior began to decline and only the three large males continued to produce milt. The experiment was terminated because natural spawning conditions were arriving.

Winter 1993-94

In the winter of 1993-94 a similar experiment was conducted. Bluegill were again collected through the ice from Lake Ovid. Bluegill were acclimated to 12°C using the same procedures. It was hypothesized that a possible factor in the failure of the previous years experiment was overcrowding in the tank. Therefore, a larger tank (1900 liter) was used to contain the same number of fish used the previous year (15 males and 15 females). A similar method of temperature and photoperiod adjustment was initiated following one month of laboratory acclimation. One week following the completion of the temperature and photoperiod adjustments, several males were identified as ripe. Females showed no signs of increased ripeness.

Banner and Hyatt (1975) administered injections of human chorionic gonadotropin (HCG) to increase the number of successful spawning inductions in female bluegill. Wilbert (1990) administered HCG to increase the rate of egg development. Following the dosage protocols of Wilbert

(1990), injections of HCG (Sigma Chemical Company, St. Louis, Missouri) were administered to seven of the fifteen females. HCG was dissolved in distilled water at 1000 I.U./0.1 ml. The solution was injected using a 1 cc syringe and 25 gauge needle (Becton Dickinson). Intermuscular injections were administered at mid-body, just above the lateral line. All 15 of the females were examined every 2 days following injection. No change was observed in any of the females (injected or not) for one week after the injection. At this time, a second injection was administered to the same 7 females. Fish were again checked every two days. Females continued to show no signs of change. Temperature and light were maintained for two more weeks with routine examinations of all fish performed once each week. Final examinations indicated no change in female ripeness. The experiment was discontinued at this time.

The design of these experiments was similar to experiments conducted by Wilbert (1990), who reported successful spawning of both males and females. In addition, Bryan et al. (1994) recently reported techniques that were successful in obtaining 100% spawning success from males and females for 12 months of the year. Bryan et al. (1994) emphasized that the two most important factors in the success of laboratory spawning were the size of the tank and number of fish per tank. The protocols used by Wilbert (1990) did not indicate the number or sex ratio of fish he

used in the 1900 liter holding tank. Bryan et al. (1994) reported obtaining a spawn every 14 days when 2 males and 4 females were placed in a 600 liter tanks. No spawning was observed in 600 liter tanks if 10 or more fish were present. This suggests that adequate space is necessary for bluegills to develop ripe gametes in the laboratory. The failure of my attempts to induce laboratory spawning may be a result of the requirement for adequate space. Bluegill were stocked at densities of approximately 2 fish /100 liters, which was twice the density used successfully by Bryan et al. (1994).

APPENDIX B

Perhaps the most important component in attempting to establish a tetraploid broodstock is the selection of the founder population. Unintentional inbreeding and genetic drift occur in cultured populations because they are small and closed. This combination can quickly destroy a population's genetic variance and increase inbreeding, which will lower productivity and increase production costs (Tave 1986). When a population is finite, the best way to describe it is not by total population but by the effective breeding number. Effective breeding number depends on several factors; the most critical are total number of breeding individuals, sex ratio, mating system, and variance of family size. Effective breeding number in a population in which mating is random is calculated by using the following formula:

$$Ne = 4(♀)(♂)/(♀)+(♂)$$

where Ne is the effective breeding number, ♀ is the number of females that produce viable offspring, and ♂ is the number of males that produce viable offspring. An

examination of the formula shows that Ne can be increased by increasing the number of breeding individuals or by bringing the population closer to a 50:50 sex ratio. Ne is inversely related to inbreeding. As Ne decreases, inbreeding increases.

Reductions in Ne can seriously affect a population's biological potential, adversely affect productivity and ruin the opportunity to improve the stock via selection (Tave 1986). Kincaid (1979) recommended that Ne be at least 500. Ryman and Stahl (1980) recommended that Ne be at least 60. Tave (1986) recommended Ne between 263 and 344 for food fish and bait fish populations and Ne between 424 and 685 for populations to be stocked in natural bodies of water.

Unfortunately, there is no single number that can be used to prevent genetic drift or inbreeding related problems from occurring in every population. Tave (1986) outlined procedures to determine minimum Ne that can be used to prevent inbreeding and genetic drift problems.

A founder population is only as useful as its own founding population. Information about a populations Ne is an important selection criterion. It does little good to acquire broodstock by sampling a population of 1,000,000 fish if that population was produced by only eight brooders. Tave (1986) suggested that care also be taken in sampling procedures. Tave (1986) suggested that broodfish be collected randomly over a wide geographic range. In the

case of bluegill, it is typical for a body of water to have several spawning areas distributed throughout the system.

Genetic marking studies performed on smallmouth bass, revealed a high degree of philopatry, suggesting that adult bass return to natal areas to spawn (Gross and Kapuscinski 1994). If other centrarchid fishes, more specifically bluegill, exhibit similar homing responses to natal spawning areas, it is likely that the bluegill in any specific spawning area are more closely related to each other than to bluegill occupying other spawning areas throughout a lake or pond system. In such a case, the best sampling approach would be to collect fish from as many different spawning areas as possible.

Although several males were used to fertilize relatively small numbers of eggs in this study, two males should be sufficient to fertilize all of the eggs from one female. Considering the amount of time and effort involved in collecting ripe adults from a natural body of water, an appropriate goal for a commercial culture operation would be to collect at least 480 ripe adults, consisting of 160 females and 320 males. Using this number of fish, Ne will equal 425, which reaches the minimum value suggested by Tave (1986) for fishes stocked into natural bodies of water.

Adult female bluegill produce between 6000 to 9000 eggs per spawning event (Garling, personal communication). Assuming similar survival rates and tetraploid yields to

those obtained in this study, 480 ripe adults, consisting of 160 females and 320 males could produce a total of 2,784 tetraploids surviving to swim up.

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