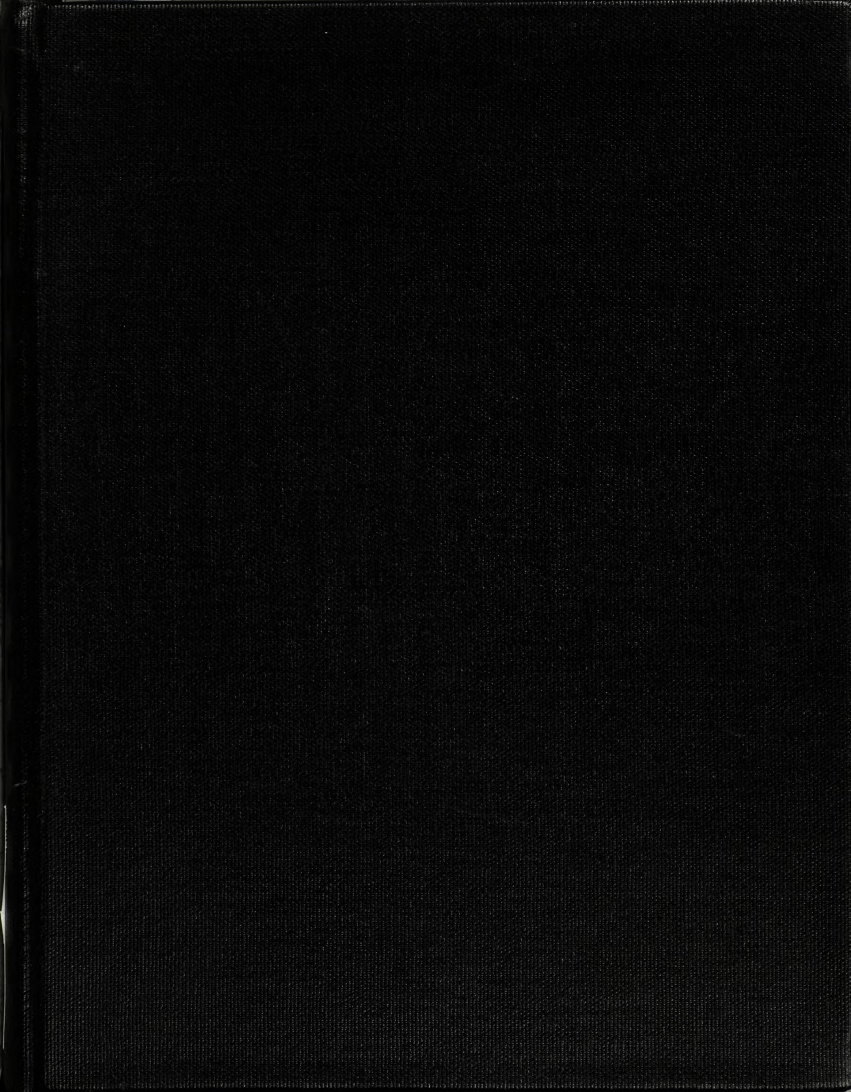




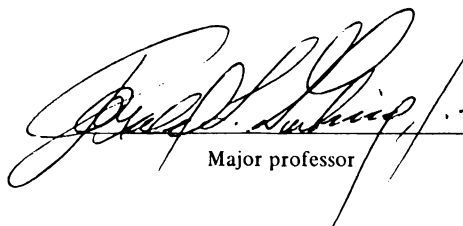


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ATTEMPTED TETRAPLOID INDUCTION IN BLUEGILL SUNFISH
(LEPOMIS MACROCHIRUS) USING HEAT SHOCKS

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Paul D. Wilbert

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Master of Science degree in Fisheries and Wildlife


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ATTEMPTED TETRAPLOID INDUCTION IN BLUEGILL SUNFISH
(LEPOMIS MACROCHIRUS) USING HEAT SHOCKS

By

Paul D. Wilbert

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTERS OF SCIENCE

Department of Fisheries and Wildlife

1990



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ABSTRACT

ATTEMPTED TETRAPLOID INDUCTION IN BLUEGILL SUNFISH (LEPOMIS MACROCHIRUS) USING HEAT SHOCKS

By

Paul D. Wilbert

Sterile triploid and fertile tetraploid bluegill have significant potential for use in fishery management and aquaculture. Experiments were conducted to develop techniques to produce tetraploid bluegill sunfish. Preliminary studies indicated that: the upper lethal heat shock temperature for bluegill eggs was 40°C for 10 minutes, bluegill could be spawned out-of-season, bluegill fry could be raised on brine shrimp nauplii, and bluegill ploidy levels could be determined by flow cytometry.

Triplicate groups of bluegill eggs were heat shocked at temperatures of 30, 35, and 40°C for 2.5, 5.0, 7.5, and 10.0 minutes. Three controls groups used. All heat shocks were initiated 30 minutes after fertilization which is approximately 5 minutes before cytokinesis. Five fish from each heat shock treatment and control replicate were analyzed for ploidy by flow cytometry. Fish tested for ploidy were 67 to 85 days old.

No tetraploid bluegill were identified from the 180 tested. Tetraploids may have been produced; but, were either in too low a number to identify by the sampling procedure or died prior to the swim up stage of development.



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I would like to thank my committee, Dr. Donald Garling, Dr. Clarence McNabb, and Dr. Patrick Muzzall for their advice and input into this project. A special thanks goes to Dr. Garling, my major professor, for his guidance and never ending patience with me.

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INTRODUCTION

Stunting of the bluegill sunfish (Lepomis macrochirus; Fam: Centrarchidae) is a problem that is often encountered in ponds and small lakes. Stunting is caused by inadequate amounts of food and/or space that lead to a decrease in individual growth rate of fish (Murnyak et al., 1984). Overpopulation is usually the cause of stunting. Potentially, polyploid bluegills could be used to decrease the population size and decrease or eliminate stunting.

A polyploid organism is one with three or more complete sets of chromosomes (Ayala and Kiger, 1984). Triploid fish have three sets of chromosomes (Ayala and Kiger, 1984) and are sterile (Purdom, 1983; Thorgaard, 1986). Tetraploid fish have four sets of chromosomes (Ayala and Kiger, 1984) and are fertile (Chourrout et al., 1986, Chourrout and Nakayama, 1987). Normal bluegill sunfish (diploid) have 48 chromosomes (Roberts, 1964); consequently a triploid bluegill would have 72 chromosomes and a tetraploid bluegill would have 96 chromosomes.

Artificially induced polyploid fish have many potential aquaculture and fisheries management applications. Extended growth and/or survival of mature fish may be expressed by



triploid fish (Thorgaard, 1986). Sterile triploid grass carp (Ctenopharyngodon idella) can be used for aquatic weed control (Wattendorf, 1986) where reproduction is undesirable. Triploid fishes may be desirable where overpopulation and stunting occur (Thorgaard and Allen, 1987).

Tetraploid fishes could be crossed with diploid fish to produce triploid fish (Chourrout et al., 1986; Chourrout and Nakayama, 1987). This method would have greater efficiency compared to direct triploid induction using shocks. Mortalities due to heat shocking and handling of triploids would be eliminated. All of the fish produced by the cross of diploid and tetraploid fish would be triploid, so undesirable diploid reproduction would be eliminated. A tetraploid broodstock can be easily maintained as tetraploid fish can perpetuate themselves by normal mating (Chourrout and Nakayama, 1987).

Polyploid bluegills could be used to control or eliminate stunting in small lakes or ponds. In new ponds, or ponds where the resident fish have been removed, sterile triploid bluegills could be stocked directly. A continued stocking program would be required to maintain the population. Tetraploid bluegills of one sex and diploid bluegills of the other sex could be stocked into these types of ponds instead to produce triploid offspring. Finally, tetraploid bluegills could be introduced into a resident population of bluegill sunfish and over time a larger



percentage of the total offspring produced would be sterile triploid bluegills. Male triploid rainbow trout (Benfey et al., 1986) and grass carp (Allen et al., 1986) have produced aneuploid sperm, and eggs fertilized with this sperm were not viable. Triploid bluegill may show spawning behavior, but fertilization by triploid sperm would yield inviable eggs and would reduce the bluegill population. Initially, artificial induced tetraploid bluegills would have to be produced to establish a tetraploid broodstock.

Polyploid bluegill have a great potential for aquaculturists as a food fish. The number of triploid bluegill stocked in a pond would be absolute. The rate of stocking triploid bluegill could be calculated so the carrying capacity of the pond would be attained when the fish reach market size. The market size of bluegill would be determined by fish processors.

Artificially induced polyploid fish have been produced by applying a temperature, pressure, or chemical shock to a fertilized egg at the appropriate time in development. In producing triploid fish, the shock must be applied early in development prior to the second meiotic division (Purdom, 1983). Tetraploidy has been induced when the shock was applied just prior to the first cellular division (Purdom, 1983). Shocks that have been used include temperature (Swarup, 1959a; Lincoln et al., 1974; Valenti, 1975; Lemoine and Smith, 1980; Thorgaard et al., 1981; Chourrout, 1982;



Benfey and Sutterlin, 1984; Solar et al., 1984; Bidwell et al., 1985; Johnstone, 1985; Arai and Wilkins, 1987; Thompson et al., 1987; Don and Avtalion, 1988), pressure (Benfey and Sutterlin, 1984; Chourrout, 1984; Lou and Purdom, 1984), and chemical (Smith and Lemoine, 1979; Refstie et al., 1977; Allen and Stanley, 1979; Refstie, 1981; Shelton et al., 1986; Johnstone et al., 1989).

A number of important preliminary studies were completed in 1988-89 prior to tetraploidy induction experiments. A pilot study was completed to determine the upper lethal temperature for bluegill sunfish eggs. This study is outlined in Appendix A. Techniques to raise bluegill fry with brine shrimp is outlined in Appendix B. Ploidy of the bluegill obtained from heat shock experiments was analyzed using flow cytometry; the techniques are presented in Appendix C. Tetraploid induction experiments were performed on the bluegill sunfish during the summer of 1989. The body of this thesis describes attempted tetraploid induction in bluegill sunfish using heat shocks.



LITERATURE REVIEW

Stunting of Bluegill Sunfish

Stunting in bluegill sunfish is caused by inadequate amounts of space and/or food that lead to a decrease in individual growth rate of fish (Murnyak et al., 1984). In fish, a marked negative correlation has been observed between population density and the rate of body growth (Smith, 1980). The density of bluegill in a given body of water will determine the size of bluegill found. Predators are necessary to lower bluegill numbers, so the population remains normal (Mittlebach, 1981).

Aquatic vegetation also has been shown to influence the level of stunting observed in lakes and ponds. As the area of submersed vegetation increased, the predation of bluegill sunfish by largemouth bass (Micropterus salmoides) decreased (Savino and Stein, 1982) and growth of bluegill sunfish decreased (Crowder and Cooper, 1982). The decrease in growth of the bluegill sunfish is due to reduced predation efficiency by the largemouth bass (Crowder and Cooper, 1982).



Reducing the number of stunted fish in lakes and ponds has increased the size of the remaining fish (Beckman, 1941) and condition factor (Beckman, 1943). The condition factor is used as an index of the length and weight of an individual fish (Piper et al., 1983). When stunted bluegill sunfish are placed into a body of water containing a normal bluegill population, they grew; but not as well as the unstunted bluegills (Murnyak et al., 1984). Potentially triploid fish could be utilized where overpopulation and stunting occurs (Thorgaard and Allen, 1987).

Natural Occurring Polyploids

Naturally occurring polyploidy has been relatively common in plants, but rarely found in animals, occurring only in lower forms such as annelids, insects, crustaceans, fish, and amphibians (Ayala and Kiger, 1984). Natural triploid fish have been identified from several species.

In salmonids, naturally occurring triploids have been found in brook trout (Salvelinus fontinalis) (see Allen and Stanley, 1978) and rainbow trout (Oncorhynchus mykiss) (see Cuellar and Uyeno, 1972; Thorgaard and Gall, 1979). Single triploid fish have been found in natural populations of California roach (Hesperoleucus symmetricus) (see Gold and Avise, 1976) and fathead minnow (Pimephales promelas) (see Gold, 1986). Naturally occurring triploids have been



reported by Schultz (1967) in a population of hybrid poeciliids (Poeciliopsis lucida X Poeciliopsis latidens). Triploid fish were probably produced by triploid eggs being elevated to a hexaploid (6N) level by an endomitotic division and the sperm stimulated the egg to develop without fertilization (Schultz, 1967). Electrophoresis could be used to confirm sperm stimulation, if a suitable isozyme marker is found. Naturally occurring triploids have also been found from the hybrid Poecilia latipinna and Poecilia mexicana (see Menzel and Darnell, 1973).

Induction of Tetraploidy

Tetraploidy in fishes has been artificially induced in two ways by applying a shock just prior to the first mitotic division of the developing egg. Tetraploid fish have been produced when the separation of chromatids (karyokinesis) was interrupted, or the separation of cytoplasm (cytokinesis) was interrupted (Chourrout, 1984). A triploid egg shocked prior to the second meiotic division will also result in a tetraploid offspring (Chourrout et al., 1986). A triploid egg has been produced by fertilizing a normal haploid (1N) egg with diploid (2N) sperm from a tetraploid male. Heat shocks may denature the spindle apparatus (Briggs, 1947; Fankhauser and Godwin, 1948) and this could lead to polyploid induction. Rieder and Bajer (1979)



showed that heat shock depolymerizes the microtubules in cultures of newt lung cells. Tetraploidy has been induced in amphibians in newts (Fankhauser, 1945; Fischberg, 1958) and frogs (Gurdon, 1959). Experiments involving tetraploid induction in fish are summarized in Table 1.

Early attempts at tetraploid induction in fish leaves some question as to whether the fish obtained were tetraploids. Valenti (1975) attempted to induce tetraploidy in Tilapia aurea using heat shocks; but appears to have induced triploidy, tetraploidy, and mosaics. Mosaics are fish with triploid and tetraploid cells. Refstie (1981) induced polyploidy in rainbow trout and concluded that the fish he produced were "probably tetraploid."

Several methods have been used to induce tetraploidy. Tetraploid fish have been produced using temperature shocks. Heat shocks have been used to produced tetraploid coldwater fish (Thorgaard et al., 1981; Chourrout, 1982) and warmwater fish (Valenti, 1975; Bidwell et al., 1985). Heat shocks also have been used to induce tetraploid rainbow trout by shocking a developing triploid egg (Chourrout et al., 1986; Blanc et al., 1987). Cold shocks have been used to produce tetraploid Tilapia aurea (see Valenti, 1975).

Hydrostatic pressure has been used extensively in inducing tetraploidy in rainbow trout (Chourrout, 1984; Chourrout et al., 1986; Myers et al., 1986). Low levels of tetraploid induction were found when hydrostatic pressure



Table 1. Summary of Tetraploid Induction Experiments in Fishes

Species	Shock Method	Primary Author and Year (1900)	
Atlantic salmon	cytochalasin b	Allen	79
brook trout	cold	Lemoine	80
channel catfish	heat	Bidwell	85
chinook salmon	pressure	Myers	86
coho salmon	pressure	Myers	86
coho x Atlantic salmon	pressure	Myers	86
rainbow trout	heat	Thorgaard	81
		Chourrout	82
	pressure	Chourrout	84
		Chourrout	86
		Myers	86
	cytochalasin b	Refstie	77
		Refstie	81
tilapia	heat	Valenti	75
	cold	Valenti	75
	pressure and cold	Myers	86



shocks were used with different salmonid species - chinook salmon (Oncorhynchus tshawytscha), coho salmon (Oncorhynchus kisutch), and the hybrid female coho x male Atlantic salmon (Salmo salar) (see Myers et al., 1986). Myers (1986) used a combination of hydrostatic pressure and cold shock to produce tetraploidy in two tilapia, Oreochromis niloticus, Oreochromis mossambicus, and their hybrid.

Induction success has varied among the different treatments. The rate of temperature shock induction has varied from 16 percent (Thorgaard et al., 1981) to 75 percent (Valenti, 1975). Hydrostatic pressure treatments produced 90 percent (Myers, et al., 1986) to 100 percent (Chourrout, 1984; Chourrout et al., 1986; Blanc et al., 1987) success in tetraploidy inducement. Treatments using hydrostatic pressure and cold shock in tilapia yielded up to 8.3 percent induction.

Survival of the shocked fish has varied by species. The survival of rainbow trout have been 10 percent (Chourrout et al., 1986), 4.1 percent (Myers et al., 1986) or approximately 30 percent when produced by heat and hydrostatic pressure shocks (Thorgaard et al., 1981; Chourrout, 1982; Chourrout, 1984; Blanc et al., 1987). Tetraploid rainbow trout induced by cytochalasin b had only a 12 percent survival rate (Refstie, 1981). Other salmonid tetraploids have had very poor survival rates, never exceeding 1.8 percent (Myers et al., 1986). Tetraploid



tilapia produced by heat shocks and cold shocks had a 50 percent and 90 percent survival rate respectively (Valenti, 1975). Myers (1986), observed up to 8.3 percent survival of tetraploid tilapia in two species and their hybrid using hydrostatic pressure and cold shock. Survival of tetraploid channel catfish was 40 percent (Bidwell et al., 1985).

Induction of Triploidy in Fish

While the induction of tetraploidy in fish is a relatively new tool for fisheries managers, triploidy has been utilized for several years. Triploidy induction experiments have provided the basis for attempts in tetraploid induction. Heat shocks have been the most successful method of induction in several species of fish (Chourrout, 1984). Triploid induction experiments in fishes have been summarized in Table 2.

Table 2. Summary of Triploid Induction Experiments in Fishes

Species	Shock Method	Primary Author and Year (1900)
Atlantic salmon	heat	Benfey 84
		Johnstone 85
	pressure anaesthetics	Benfey 84
		Johnstone 89
brook trout	cold	Lemoine 80
brown trout	heat	Arai 87
carp	cold	Gervai 80
channel catfish	cold	Wolters 81
	heat	Bidwell 85
chinook salmon	heat	Utter 83
		Westerhof 88
		Spruell 89
coho salmon	heat	Utter 83
grass carp	heat	Thompson 87
pink salmon	heat	Utter 83
rainbow trout	heat	Chourrout 80
		Thorgaard 81
		Lincoln 83
		Lou 84
		Solar 84
		Bye 86
		Thorgaard 86
		Lou 84
		Shelton 86
	pressure nitrous oxide	
stickleback	heat	Swarup 59a
	cold	Swarup 59a
tilapia	heat	Valenti 75
		Don 88
	cold	Valenti 75
		Don 88



Ploidy Determination

Karyotyping

Karyotyping has been the most common way of determining the ploidy level of a fish. A karyotype is the characterization and analysis of a chromosome complement at metaphase within the nucleus of a given species (Blaxhall, 1983). Kligerman and Bloom (1977) developed a simpler chromosome preparation technique for karyotyping. The technique has been used in the identification of polyploids in 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 (Ctenopharyngodon idella X Hypophthalmichthys nobilis) (see Cassani et al., 1984), and various tilapia species (Myers, 1986).

Staining of blastodiscs that have been removed from the egg has been used in determining ploidy of fertilized eggs (Lincoln et al., 1974; Refstie et al., 1977). Baksi and Means (1988) developed an inexpensive technique of preparing larval fish for chromosome examination. They noted however, as well as Kligerman and Bloom (1977), that multiple samples would be very time consuming. Another difficulty in studying fish chromosomes by karyotyping techniques is their small size and the relatively large



numbers of chromosomes present (Blaxhall, 1983).

Cytophotometric DNA Measurement

Another method of determining ploidy levels in fish has been the use of a microdensiometer to measure the amount of DNA in the nucleus cytophotometrically. Blood was Feulgen stained (Johnstone, 1985) and the DNA content was measured in the erythrocytes using a microdensiometer (Gervai et al., 1980). This technique has been used successfully in triploid carp, Cyprinus carpio (Gervai et al., 1980), rainbow trout (Shelton et al., 1986) and triploid Atlantic salmon (Johnstone, 1985; Johnstone et al., 1989).

Comparison of Erythrocyte Volumes

Significant differences have been found between the nuclear volume of erythrocytes in polyploid and diploid fish (Swarup, 1959a; 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). Erythrocyte volume was determined by the equation $V = (4/3)ab^2$, where (a) equalled the major semiaxis and (b) equalled the minor semiaxis of the erythrocyte (Valenti, 1975). Lou and Purdom (1984) measured erythrocytes, cartilage, brain, and epithelial cells; but,



obtained their best results using erythrocytes. When compared to karyotyping, erythrocyte volumes were less tedious and highly (92.65 percent) accurate (Wolters et al., 1982).

Use of Coulter Counter and Channelyzer

The use of a Coulter Counter and Channelyzer to measure erythrocyte volume has proven to be a rapid and accurate method for identification of polyploids (Benfey et al., 1984). While a Coulter Counter and Channelyzer are expensive, they are cost efficient, since large numbers of fish can be done rapidly (Wattendorf, 1986). In Florida, all triploid grass carp must have their ploidy confirmed by the Coulter Counter or similar instrument before they can be stocked for aquatic weed control (Cassani and Canton, 1986; Thompson et al., 1987).

The Coulter Counter can be used to measure the actual erythrocyte volume of any aged fish. 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. The accuracy of a small number of chromosome counts or erythrocyte nuclear volume measurements to identify mosaic polyploid fish has been



questioned (Benfey et al., 1984).

Flow Cytometry

Measurement of erythrocyte DNA content by flow cytometry has been a very practical method for rapid and accurate identification of polyploid fish (Thorgaard et al., 1982). Erythrocytes were stained with a fluorescent stain. Stained single cells were suspended in an aqueous solutions and delivered, single file, to a signal detection center by vacuum or air pressure at rates of thousands of cells per second. As each cell traversed the laser beam, it scattered fluorescent light which was filtered and collected by a fluorescent detector. The light signals were converted into electric signals, measured, digitized, and sent to a computer for analysis, display and storage (Downing et al., 1984).

Two fluorescent stains have been used in the analysis in fish DNA: 4'-6-diamidino-2 phenylindole (DAPI) (Thorgaard et al., 1982; Utter et al., 1983; Solar et al., 1984) and propidium iodine (Allen, 1983). Fish species that have been tested by flow cytometry have included 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; Johnson et al., 1986), chinook salmon (Johnson et al., 1986; Westerhof,



1988; Spruell, 1989), grass carp (Allen et al., 1986) and hybrid grass carp X bighead carp (Allen, 1983). Johnson et al. (1984) found that the flow cytometer is more accurate (100 versus 89 percent) than the Coulter counter in ploidy measurements.

Miscellaneous Methods

Electrophoresis has been used in determining the ploidy of grass carp (Wiley and Wike, 1986) and soft-shell clams (Allen et al., 1982). Silver staining has been used in identifying triploid fish cells (Phillips et al., 1986). Chinook salmon, coho salmon, and rainbow trout ploidy levels were determined by counting the number of nucleoli per cell. Diploid fish had one or two nucleoli per cell while triploid fish had one, two, or three nucleoli per cell. Multiple cell samples were since only triploid fish had three nucleoli per cell (Phillips et al., 1986). This method does not provide absolute identification of diploid fish, and would limit its use in most applications.

MATERIALS AND METHODS

In a pilot study, it was determined that the upper lethal temperature for heat shocking bluegill eggs was 40°C for 10 minutes (Appendix A). This was comparable to tetraploid induction experiments done with tilapia (Valenti, 1975) and channel catfish (Bidwell et al., 1985). Tetraploidy induction has been induced when a shock is applied to eggs just prior to the first cleavage (Thorgaard, 1986). The first cleavage has been reported to occur 35 minutes after fertilization in the bluegill sunfish (Morgan, 1951). Shocking of bluegill eggs beginning 30 minutes after fertilization should result in the disruption of the first cleavage. Shocks lasting up to ten minutes cover the development of the bluegill egg through first cleavage.

Spawning bluegill were collected by hook and line from Lake Lansing (near Haslett, Michigan) from June 28 to July 28, 1989 and transported to the Michigan State University Aquaculture Laboratory (East Lansing, Michigan) in aerated 163 liter coolers. Bluegill were identified in the field and verified at the laboratory (Eddy and Underhill, 1978). The bluegill were transferred to 38 liter aquaria maintained at 20°C, 0.5 liter/minute water flow and equipped with an

external standpipe (Garling and Wilson, 1976) to maintain a constant water level.

Bluegill were hand spawned after being allowed to acclimate at least 30 minutes. Female bluegill were considered ripe and ready to spawn when gentle squeezing of both sides of the abdominal area resulted in egg flow from the genital pore. Female bluegill were dried around the genital opening and eggs were stripped onto a clean, dry watch glass. Bluegill eggs were stripped from the fish by applying firm, gentle pressure to both sides of the abdominal area of the female bluegill. One or two female bluegill were stripped into each watch glass depending on the amount of eggs obtained per female. Since percentage of tetraploid bluegill surviving to 3-4 cm in length was to be analyzed, the actual number of bluegill eggs shocked were not counted. Egg batches were discarded if the eggs had malformed oil globules, physical deformities, or cloudiness. Malformed oil globules indicated under-ripeness and cloudiness indicated over-ripeness (Banner and Hyatt, 1975).

Male bluegill were dried around the genital opening and were spawned by applying firm, gentle pressure to both sides of the abdominal area. Milt was collected using a 5 3/4 inch pasteur pipet and pipet bulb (VWR Scientific). Milt viability was confirmed by taking a small sample of the milt collected, making a smear on a slide, and examining it under a compound microscope (American Optical) at 450X power. If

individual, mobile sperm were observed, the milt was considered viable and added to the eggs. Inviabile milt was discarded. Viable milt from at least two male bluegill was added to each batch of bluegill eggs to ensure fertilization. Stirring with a finger mixed the milt and the eggs. When the milt was added to the eggs, a stopwatch (Micronta) was started to time egg development.

Stripped female bluegill were placed in a concrete pond (10' x 15' x 12' deep) located adjacent to the laboratory for use in future experiments. Male bluegill were held in a 1900 liter holding tank maintained at 20°C and 2 liter/minute flow. Male bluegill could be hand spawned repeatedly throughout the experiment period.

One minute after fertilization, 20°C water was added to the bluegill eggs and milt to water activate the bluegill eggs (Piper et al., 1983). Fertilized bluegill eggs were maintained at 20°C to ensure accurate timing of egg development for heat shocking. At thirty minutes post-fertilization, heat shocks were applied to the bluegill eggs.

Heat shocks were performed at 30, 35, and 40°C for 2.5, 5.0, 7.5, and 10.0 minutes (Figure 1). Thirty minutes after fertilization the bluegill eggs were submersed in a hot water bath (Figure 2). The temperature of the hot water bath was maintained, at heat shock temperatures (+/- 0.25°C) by a 75 watt submersible aquarium heater (Dan-sea).

Figure 1. Temperature and duration regime of heat shocks applied to bluegill sunfish eggs.

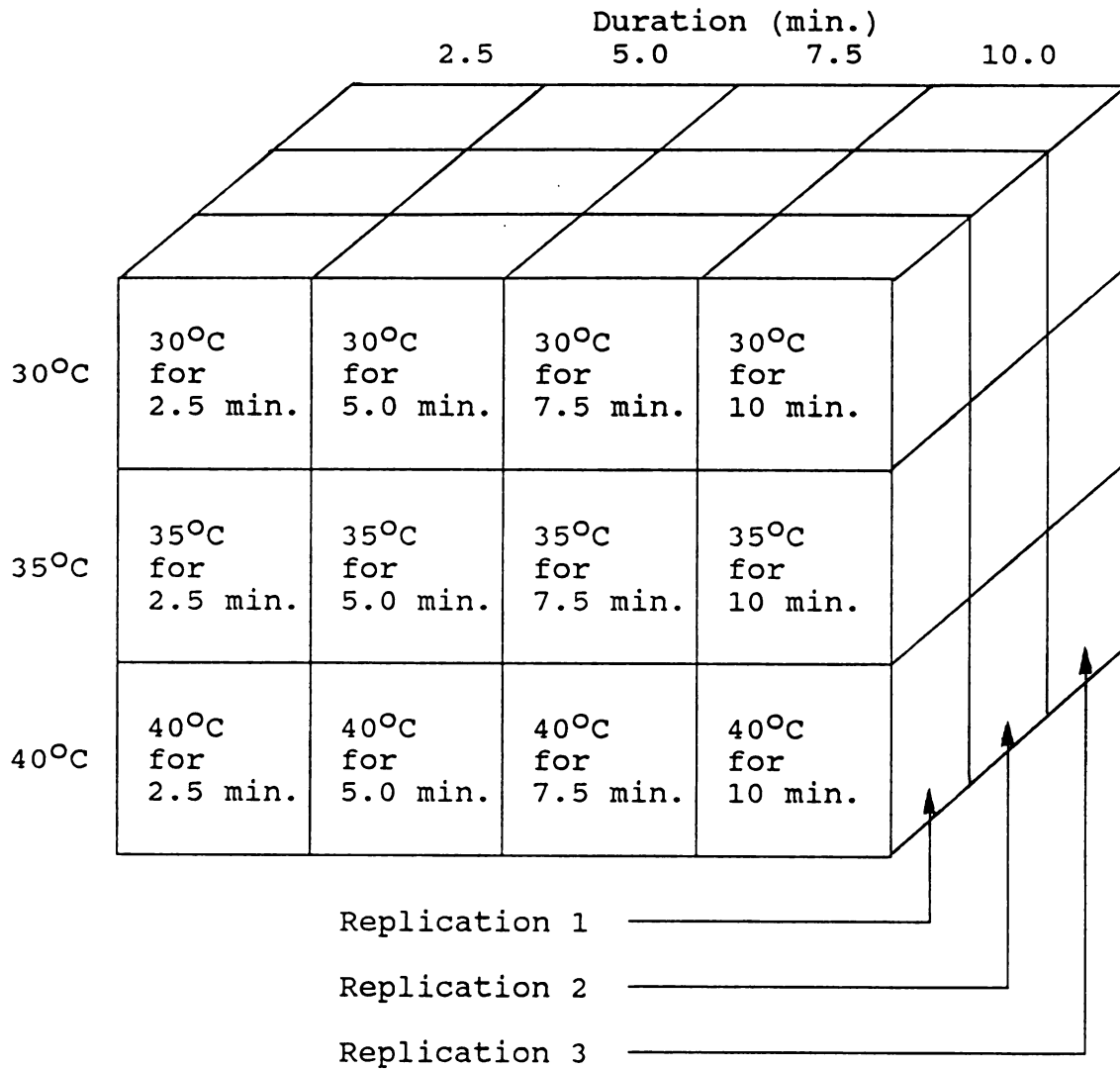
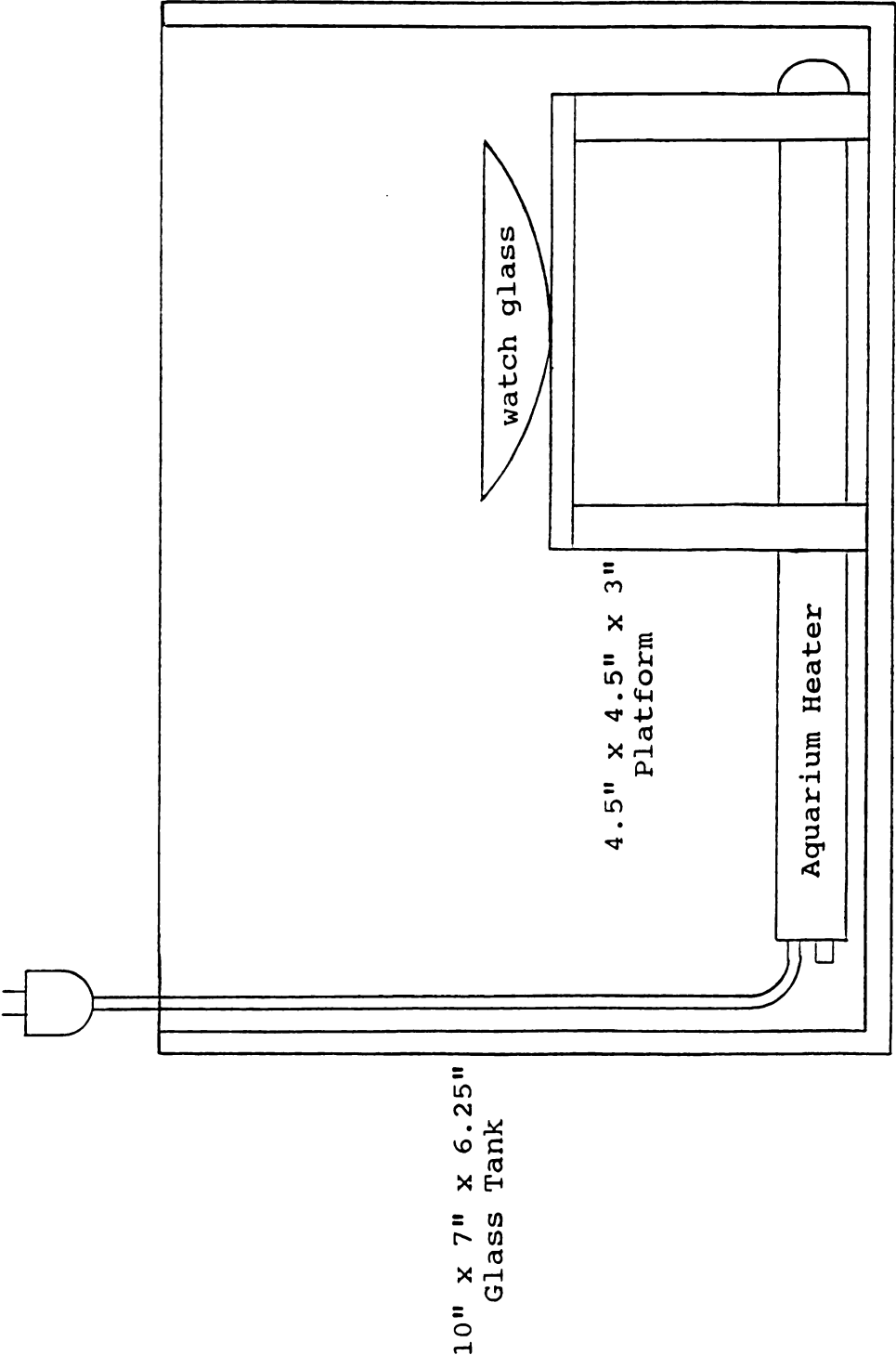


Figure 2. Hot water bath used to heat shock bluegill sunfish eggs.



Bluegill eggs were heat shocked at the appropriate temperature for the allotted amount of time and then carefully transferred without acclimation to a 38 liter aquaria maintained at 26°C, 0.5 liter/minute flow and equipped with an external standpipe (Garling and Wilson, 1976) to maintain a constant water level. Control groups of eggs were held at 20°C for 35 minutes after fertilization and then placed into an aquaria. One replicate of each individual treatment was completed before additional replicates were completed. A total of three replicates were performed for each treatment. Three control groups were used, each being performed before new treatment replicates. A total of 36 individual bluegill egg heat shocking treatments and three controls were completed (Figure 1).

Bluegill eggs were incubated at 26°C to speed development and decrease the potential growth of fungus on dead bluegill eggs. Secondary temperature shock, the return from hot to cold water, was minimized and chemical treatment of bluegill eggs for fungus was unnecessary. Morgan (1951) noted that bluegill eggs hatched between 32 and 62 hours after fertilization when incubated at 72°F (22°C). At 26°C, hatching time would possibly be accelerated and should occur between 30 and 62 hours after fertilization.

Bluegill fry were found to swim up nine to ten days after fertilization when incubated at 20°C (Appendix A). Fry needed to be fed immediately at swim up. It had been

estimated that at 26°C it would take six to seven days after fertilization for the bluegill fry to swim up. Six days after fertilization the bluegill fry were fed newly hatched brine shrimp nauplii and continued to be fed four times daily until tested for tetraploidy (Appendix B).

Aquaria water temperature was decreased from 26°C to 20°C after swim up over a six hour period. Water temperature was monitored in the morning and maintained at 20°C. Photoperiod was maintained at 18 hours light and 6 hours dark by an automatic timer (Intermatic Incorporated).

Beginning on the fourth day of feeding, aquaria were siphoned each morning to remove feces and uneaten brine shrimp which accumulated the previous day. Solid wastes were not removed by siphoning until three days after the first feeding due to the small size of the bluegill fry and their susceptibility of being caught in the siphon action.

Approximately 65 days after fertilization, bluegill attained a size suitable for tetraploidy analysis. Bluegill needed to reach a size where a half drop of blood could be drawn. Bluegill to be tested for tetraploidy were anesthetized using tricane methane sulfonate (Finquel MS-222, Argent Chemical Laboratories). Blood was sampled from individual bluegill by cardiac puncture using a 1 cc syringe with a 25 gauge needle (Becton Dickinson). Blood samples were taken from five bluegill from each heat shock treatment replicate and were tested for ploidy using flow cytometry

(Appendix C).

The procedure for testing bluegill blood using flow cytometry was similar to the procedure used for testing chinook salmon (Oncorhynchus tshawytscha) (see Spruell, 1989). Fifteen bluegill were sampled from each heat shock treatment group. If tetraploidy had been detected from any heat shock treatment replicate an additional ten bluegill would have been tested to determine the percentage of tetraploid fish in that treatment group. Treatments containing tetraploid bluegill would have been retained and raised for future experiments.



RESULTS

As expected based on preliminary experiments, no bluegill hatched from the 40°C heat shock treatment groups at ten minutes duration. Qualitative observations were made on the egg and fry survival of the remaining treatments. Bluegill egg mortality appeared to increase as the heat shock temperature and duration increased. Survival of eggs in the 30 and 35°C treatments for 2.5 minutes appeared to be comparable to controls. Hatching of bluegill eggs occurred between 30 and 48 hours after fertilization. Heat shocked eggs treated at 35°C for 7.5 minutes hatched in relatively large numbers; however, two days after hatching large die offs of the bluegill fry occurred in two of the three treatments. Mortality data was not collected, since this study was concerned with the percent of tetraploid bluegill surviving to 3-4 cm in length. At least five bluegill survived to be analyzed for ploidy in all remaining treatments.

After swim up, mortality was extremely low for all groups. Swim up fry were observed to feed aggressively on newly hatched brine shrimp nauplii. Brine shrimp nauplii were offered in large quantities and the majority were not

eaten and accumulated on the bottom of the tank. Daily siphoning kept the water quality suitable for the growing fry. Few bluegill were killed by siphoning.

Blood samples from bluegill were tested for tetraploidy on 25, 26, September and 5, 6, 9, 10 October, 1989, using flow cytometry. Blood samples had been taken from 65 day old bluegill (Appendix A). Some bluegill were held past 65 days and they were tested with heat shock treatment groups performed later in the summer. Bluegill were 67 to 85 days old when analyzed. Five fish were sampled from each of the 33 treatments and 3 controls. This would allow a detection of tetraploidy at 6.66 percent (1 out of 15) for any heat shock treatment. No tetraploid bluegill were found in 180 bluegill sampled. Diploid bluegill blood histogram peaks were consistently 25-30 units of DNA fluorescence and chinook salmon blood (internal standard) was 82-92 units of DNA fluorescence.

Some bluegill were retained after ploidy analysis and fed a ground commercial trout feed (Zeigler, 5/32 inch pellet). After four to five days the bluegill began to feed aggressively on this feed.

DISCUSSION

Several procedural problems were encountered while conducting this study. Completion of the pilot study to determine the upper lethal heat shock for bluegill eggs was delayed when ripe bluegill were not available during the summer of 1988 due to record high temperatures (Appendix A). Many bluegill were collected, but few were ripe. Techniques were developed during the winter of 1988-89 to induce bluegill to spawn out of season (Appendix D) and the pilot study was completed prior to the spring of 1989. During the pilot study, zooplankton was not a reliable feed source for bluegill sunfish in the laboratory. Zooplankton were collected from ponds behind the laboratory and from Lake Ovid. Zooplankton were available to the bluegill fry, but no feeding was observed. It was speculated that the zooplankton were too large to be eaten by bluegill fry. During the winter of 1988-89 bluegill were successfully raised on newly hatched brine shrimp nauplii (Appendix B).

Unfortunately, no tetraploids were found in the ploidy analysis of 165 fish from 36 heat shock treatments. Tetraploid bluegill may have been produced, but died prior to ploidy analysis. The survival of tetraploid salmonid eggs to hatching has been very low (Thorgaard et al., 1981;

Chourrout, 1982; Chourrout, 1984; Myers et al., 1986; Chourrout et al., 1987). Tetraploid tilapia were subvital, showed abnormalities in development, and died within seven days after hatching (Myers, 1986). Ploidy determination at an earlier time in development would be desirable to identify effective tetraploid inducing shocks. Development of a technique to determine ploidy of bluegill tissue samples using flow cytometry would be the next logical step for future research in this area. Thorgaard et al. (1982) suggested that fish tissue treated with non-ionic detergent may be analyzed by flow cytometry. Flow cytometry is clearly the most accurate and time efficient methods of ploidy analysis available.

Death of tetraploid fish may be due to abnormalities caused by shocking. Myers (1986) suggested that shocks applied prior to cytoplasmic division (cytokinesis) leads to developmental abnormalities in the egg and fry. The abnormalities could be attributed to aneuploidy in the cells of the developing egg (Myers et al., 1986). Aneuploidy is the condition of a cell in which one or more whole chromosomes of a normal set are present more than once (Ayala and Kiger, 1984). Mortalities of tetraploid fish have also been associated with yolk resorption (Chourrout et al., 1986).

Tetraploid bluegill could have been produced; but failed to survive. Heat shock treatment of bluegill eggs of



35°C for 7.5 minutes produced a high number of hatching fry, but few survived to swim up. The surviving bluegill were diploids; perhaps the moribund bluegill had been tetraploids. The moribund fish were not tested as percent tetraploid induction at 65 days of age was desired. The heat shock treatment of 35°C for 7.5 minutes could be repeated and a tissue sample from the newly hatched fry analyzed using flow cytometry. Slower cooling of the bluegill eggs after shocking could reduce mortality associated with secondary temperature shock. While survival of tetraploids to swim up has been low, survival after swim up has been comparable to diploid fish (Chourrout, 1984; Chourrout et al., 1986).

Heat shocks have been the most common shock used in tetraploid induction, and should have produced tetraploid bluegill. Perhaps the high temperatures were lethal to induced tetraploid eggs. Potentially pressure shocks could be used to prevent these mortalities. Pressure shocks have been efficient in inducing tetraploidy in rainbow trout (Chourrout, 1984), but these experiments have been difficult to repeat and further tetraploid induction experiments using pressure shocks have been abandoned in favor of heat shocks (Seeb, pers. comm.). Expensive equipment is needed for pressure shocks and only a small number of eggs can be shocked at one time. Cold shocks would be another possibility for tetraploid induction in bluegill.



Myers (1986) suggests that tetraploids shocked at karyokinesis may have a better survival than those shocked at cytokinesis. Possibly the bluegill that died in the 35°C heat shock treatment for 7.5 minutes were tetraploids produced by interruption of cytokinesis. An earlier shock in development may inhibit karyokinesis in bluegill and produce viable tetraploids.

It has been argued that time in development is important when inducing tetraploidy. Myers (1986) suggests that proper timing is essential to ensure that tetraploid induction occurs at karyokinesis and not at cytokinesis. Tetraploids induced at karyokinesis may exhibit better survival to swim up than fish induced at cytokinesis (Myers, 1986). The optimal induction time may vary with eggs from female to female (Chourrout et al., 1986) and from fish collected in different water temperatures. Nearly ripe bluegills could be held in the laboratory for several days prior to hand spawning to allow acclimation and The temperature of the fish brought into the laboratory may have an effect on the timing of egg development. However; other researchers have noted that precise timing of egg development was unnecessary for the induction of tetraploidy (Thorgaard, 1981; Bidwell et al., 1985).

Tetraploidy may have been induced in this study with bluegill sunfish. First cleavage of bluegill sunfish eggs has been observed at 35 minutes post fertilization (Morgan,

1951). Consequently, the heat shock treatments performed at 30 minutes post fertilization should have produced tetraploid bluegill. Survival of tetraploid bluegill produced by heat shock appears to be less than 6.66 percent by our analysis.

Bluegill blood sample preparation techniques were developed during the winter of 1987-88. Ploidy determination of chinook salmon blood has been utilized for several years at Michigan State University using flow cytometry (Westerhof, 1988; Spruell, 1989). Analysis of bluegill blood by flow cytometry required only a few modifications (Appendix C).

CONCLUSIONS

The aquaculture and fisheries management potential for polyploid bluegill are too great to abandon future induction experiments. Tetraploidy analysis of bluegill fry heat shocked at 35°C for 7.5 minutes should be attempted first. Timing of developmental stages over a range of temperatures up to first cleavage would identify karyokinesis; therefore, increase precision of shocking and potentially higher survival of tetraploid bluegill. Tetraploid bluegill would have many applications, and tetraploid induction experiments should be continued. Cold shock treatments seem like a logical low cost method to evaluate for induction of tetraploidy in bluegill sunfish. Pressure shocks may hold more promise, but may be cost prohibitive for researchers and potential future users of the technology.

APPENDICES

APPENDIX A

The maximum temperature that could be used to heat shock bluegill sunfish eggs for ten minutes was determined prior to initiating tetraploidy induction experiments. In other warm water fishes, researchers have used heat shock temperatures of 38°C to 43°C (Valenti, 1975; Bidwell et al., 1985) to induce tetraploidy. Bidwell et al. (1985) noted that heat shocks above 41°C lasting longer than one minute were fatal to developing channel catfish eggs. Trial heat shocks were conducted at 40°C for 7.5 minutes and 35°C for 5.0 minutes to observe the lethal effects of heat shocks on bluegill eggs. Controls were run to insure that mortality was caused by the heat shock exclusively.

MATERIALS AND METHODS

Bluegill were collected by hook and line from Lake Ovid (Sleepy Hollow State Park near Ovid, Michigan) from June 24 to July 14, 1988 and transported to the Michigan State University Aquaculture Laboratory (East Lansing, Michigan) in aerated 163 liter coolers. Bluegill were identified in the field and identification was confirmed at the laboratory (Eddy and Underhill, 1978). Bluegill were transferred to 38 liter aquaria maintained at 20°C, 0.5 liter/minute flow, and

equipped with an external standpipe (Garling and Wilson, 1976) to maintain a constant water level. The bluegill were acclimated to these conditions for at least 30 minutes. Due to record high summer temperatures, a low number of spawning bluegill were collected in summer 1988. Additional bluegill were artificially spawned (Appendix D) during winter 1988-89 to complete this pilot experiment.

Bluegill were spawned and eggs were heat shocked as described above, except that the bluegill eggs were placed in aquaria maintained at 20°C. Heat shock treatment durations were limited to 5.0 and 7.5 minutes at 35 and 40°C. Two replications were completed for each heat shock treatment. Three controls were used to ensure that mortality was caused by heat shock exclusively.

Zooplankton was collected from a pond adjacent to the laboratory and from Lake Ovid and offered to bluegill fry in summer 1988. In winter 1988-89, bluegill fry were fed newly hatched brine shrimp nauplii (Appendix B). Valenti (1975) had success raising newly hatched tilapia on brine shrimp nauplii.

Bluegill fry were raised to a size where a small blood sample could be taken for ploidy analysis by flow cytometry (Appendix C). Five bluegill were analyzed from each treatment replicant. If tetraploid bluegill were found in a treatment, ten additional bluegill would have been tested to determine the percentage of tetraploid fish in that heat

shock treatment. Tetraploid bluegill would have been retained and raised for further experiments.

RESULTS

Qualitative observations were made on the survival of heat shock treatment groups. Survival to hatching was estimated to be less than ten percent with bluegill eggs that were shocked at 40°C for 7.5 minutes. Bluegill eggs shocked at 35°C for 5.0 minutes survived comparably to controls. Swim up occurred 9 to 10 days after fertilization at 20°C. All bluegill that were offered zooplankton died within 4 days after swim up (Table A1). No bluegill offered zooplankton were observed feeding and it was speculated that the zooplankton were too large to be eaten by bluegill fry. Use of progressively smaller meshed nets may have captured adequately sized zooplankton for bluegill to eat, but the use of these nets would be time consuming.

Two of three aquaria of bluegill fed brine shrimp nauplii survived and were tested for tetraploidy by flow cytometry (Appendix C) on April 17, 1989. A half a drop of blood was necessary to analyze bluegill ploidy. Bluegill of 3 cm or longer, were suitable for blood sampling. All five surviving bluegill from the 40°C for 7.5 minute treatment and five control bluegill were tested for tetraploidy. All ten bluegill tested were diploid.



Table A1. Outline of pilot experiment to determine the upper lethal temperature of bluegill eggs.

Temperature (°C)	Treatment		Date	Feed Type
	Duration (min.)			
20	control	6/24/88	plankton	
40	7.5	6/24/88	plankton	
35	5.0	7/01/88	plankton	
20	control	7/14/88	plankton	
20	control	1/22/89	brine shrimp	
40	7.5	2/11/89	brine shrimp	
35	5.0	3/14/89	brine shrimp	

CONCLUSIONS

A heat shock of 40°C applied for 10 minutes should be lethal to bluegill sunfish eggs and should be the greatest temperature and longest duration used to heat shock eggs. Zooplankton was unsuitable for feeding newly hatched bluegill fry, and newly hatched brine shrimp should be used. Zooplankton appeared to be too large for the bluegill fry to eat. It was also determined that bluegill could be tested for ploidy when they reached a size about 3 cm in length; which is approximately 65 days after fertilization.

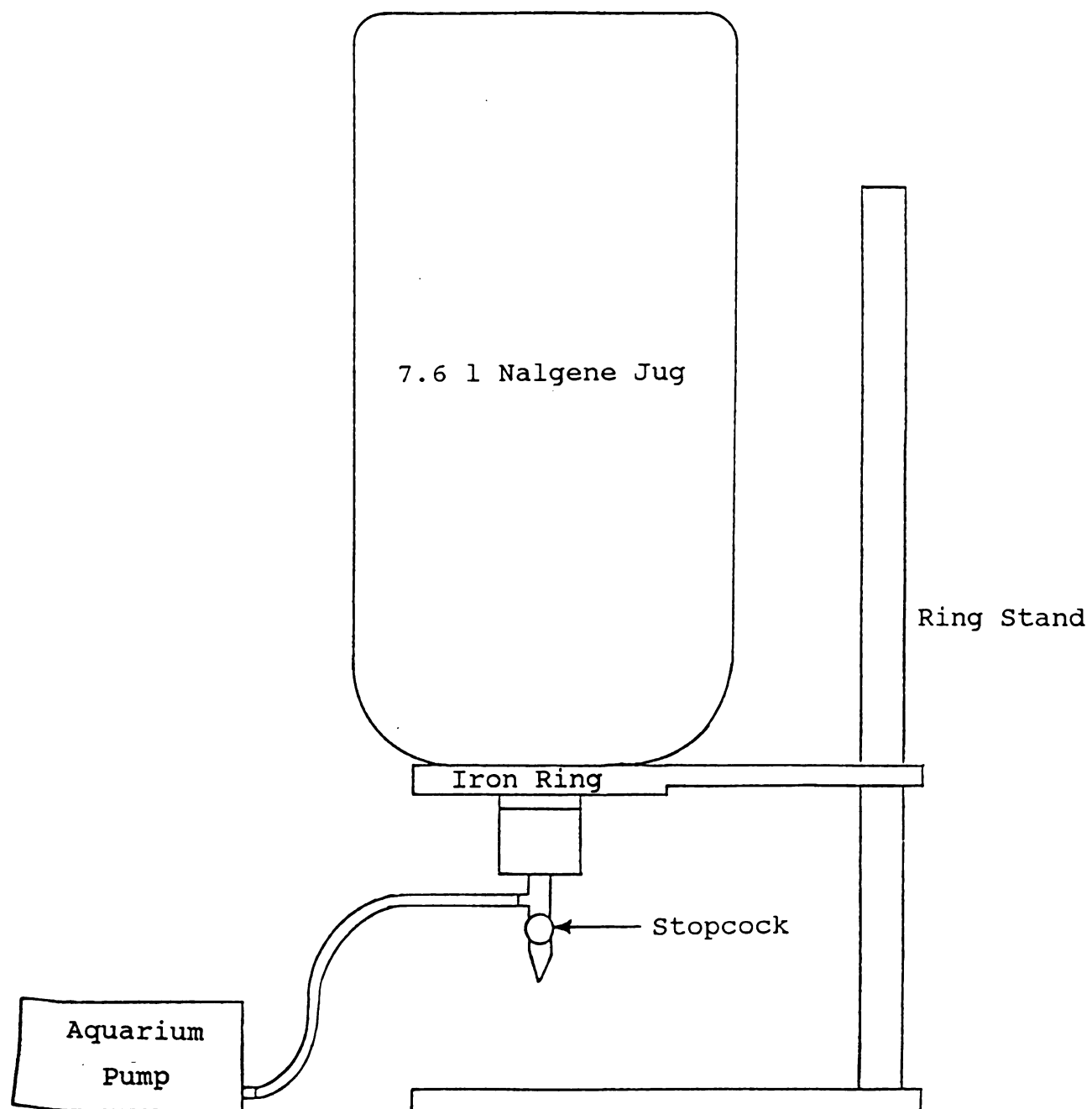
APPENDIX B

In the pilot experiment used to determine the upper lethal heat shocking temperature for bluegill eggs, plankton were unsuitable for raising bluegill fry (Appendix A). Valenti (1975) used newly hatched brine shrimp nauplii to feed tilapia. Bluegill fry were successfully raised on brine shrimp nauplii. The following is a description of how bluegill fry were raised on brine shrimp nauplii in our laboratory.

Fishes must feed at the swim up stage of development. Swim up occurs when a fish absorbs all of its yolk sac, begins swimming to the surface of the water column, and begins eating. Bluegill fry reached the swim up stage seven days after fertilization when incubated at 26°C. Newly hatched brine shrimp nauplii were used to feed the bluegill fry. The brine shrimp nauplii must be newly hatched as brine shrimp nauplii grow rapidly and become too large to be eaten by the first feeding bluegill fry.

Brine shrimp nauplii were raised in modified 7.6 l Nalgene jugs (Figure B1). Each jug cap was fitted with a t-adaptor with stopcock. An air hose was attached to the t-adaptor above the stopcock. Half of the jug bottom was cut out so brine shrimp eggs and water could be added. The jugs were inverted and placed on an iron ring that was attached to a ring stand. Air was supplied by an aquarium pump

Figure B1. Brine shrimp incubation unit.



(Second Nature Whisper 1000). Seven liters of water containing seven tablespoons of salt were added to the jug. The water pump was started and the appropriate amount of brine shrimp eggs were added. Brine shrimp eggs (San Francisco Bay Brand) were weighed on a balance (Fisher Scientific, Model 7303DA) to the nearest tenth of a gram. Water temperature was maintained between 27°C and 30°C. Artificial light was maintained for 24 hours per day. At these temperatures, brine shrimp eggs would hatch at 24 hours.

Each tank of bluegill fry was fed four times daily at 8:00 a.m., 11:30 a.m., 3:00 p.m., and 6:00 p.m. Four jugs were utilized to ensure that newly hatched brine shrimp nauplii were available at each feeding. Each tank of bluegill fry was fed the equivalent of 0.9 grams dry weight brine shrimp eggs each feeding. The air source to the jug being used to feed the bluegill fry was turned off 2-3 minutes before feeding. This allowed the brine shrimp nauplii to settle to the bottom. Brine shrimp nauplii were drained onto a piece of tightly woven cotton cloth by opening the stopcock on the bottom of the jug. The cloth would allow the saltwater to pass through while retaining the brine shrimp nauplii. The brine shrimp nauplii would be rinsed with freshwater and added to the tank of bluegill fry to be fed. Immediately following feeding the bluegill a new brine shrimp culture would be started for the following day.

During several days the air temperature, and consequently the water temperature of the brine shrimp culture rose significantly above 30°C (up to 38°C). Under these circumstances the starting time of the new brine shrimp culture was delayed, as the amount of time necessary for hatching the brine shrimp eggs was reduced. It is extremely important that the brine shrimp nauplii are newly hatched, if the brine shrimp eggs hatch 3-4 hours early they may be too large for the bluegill fry by feeding time, especially if the bluegill fry are young.

APPENDIX C

The procedure used for testing the ploidy of bluegill blood was very similar to the procedure used at Michigan State University for testing the ploidy of chinook salmon (Oncorhynchus tshawytscha) (see Westerhof, 1988; Spruell, 1989). This procedure has been used in testing experimental chinook salmon, and triploid chinook salmon for the Michigan Department of Natural Resources. Modified techniques were developed for bluegill blood during winter 1987-88 and the procedure is outlined below since it has not been used for bluegill sunfish.

The procedure for preparing bluegill red blood cell samples was modified from Spruell (1989) and is summarized in Tables C1 and C2. Bluegill ploidy was determined using an Ortho Diagnostics Systems, Inc. Model 50-H dual laser Cytofluorograph located in Giltner Hall on the MSU campus. An Ortho 2150 computer system was coupled to the cytofluorograph and the Ortho Cytofluorograph Analysis for Cellular DNA Content of Fixed Cells with DNA Doublet Discrimination program was used to analyze the data. Bluegill red blood cell samples were run at an argon-ion laser setting of 488 nm with a 0.5 W output.

Pulse-height histograms were generated by an Ortho 2150 computer system, coupled to the cytofluorograph, based on DNA measurements from 10,000 cells per bluegill blood



Table C1. Steps in the cell staining technique used to determine ploidy level in bluegill sunfish.

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



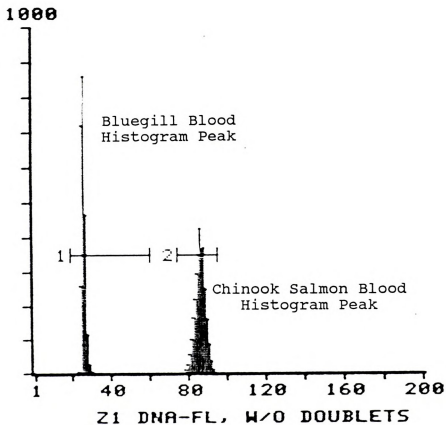
Table C2. Composition of reagents used in flow cytometric determination of ploidy level in bluegill sunfish.

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

sample. Chinook salmon blood was used as an internal standard to analyze bluegill red blood cells. If histogram peaks shift, an internal standard will discriminate accidental shifts from shifts due to a tetraploid bluegill samples. The flow cytometer was adjusted so that peaks resulting from chinook salmon blood ran at 85-90 units of DNA fluorescence and diploid bluegill peaks ran at 25-28 units of DNA fluorescence (Figure C1). Tetraploid bluegill peaks were expected to run at 48-52 units of DNA fluorescence. The position of peaks may vary slightly due to sample preparation or machine settings. A known diploid bluegill blood sample was run before experimental bluegill blood samples were run to establish the peak position of diploid bluegills. This peak, along with the chinook salmon blood internal standard, were compared to the test bluegill blood sample.



Figure C1. Data of diploid bluegill and chinook salmon blood as generated by the Ortho 2150 computer.





APPENDIX D

During the summer of 1988, the upper lethal temperature limit for heat shocking bluegill eggs was determined (Appendix A). Unfortunately, only a small number of bluegill eggs were collected due to record high summer temperatures and the pilot experiment could not be completed. Only three treatments of eggs were obtained. Three additional treatments were needed. Tetraploid induction experiments were scheduled for the summer of 1989, so it was desirable to complete the pilot experiment prior to the spring of 1989. Attempts were made in the winter of 1988-89 to induce bluegills to spawn in our laboratory using modified techniques of Banner and Hyatt (1975). Bluegill were held at 13°C, while Banner and Hyatt held fish at 23°C. Female bluegill were given a 1000 I.U. injection of human chorionic gonadotropin (HCG), while Banner and Hyatt used a 400 I.U. injection.

Bluegills used for artificial spawning experiments were caught by hook and line from Lake Ovid (Sleepy Hollow State Park, near Ovid, Michigan) during winter 1987-88. Bluegills were transported back to the Michigan State University Aquaculture Laboratory (East Lansing, Michigan) in 163 liter aerated coolers. Bluegills were identified in the field and identifications were confirmed at the laboratory (Eddy and Underhill, 1978). Bluegill were placed in a 2015 liter

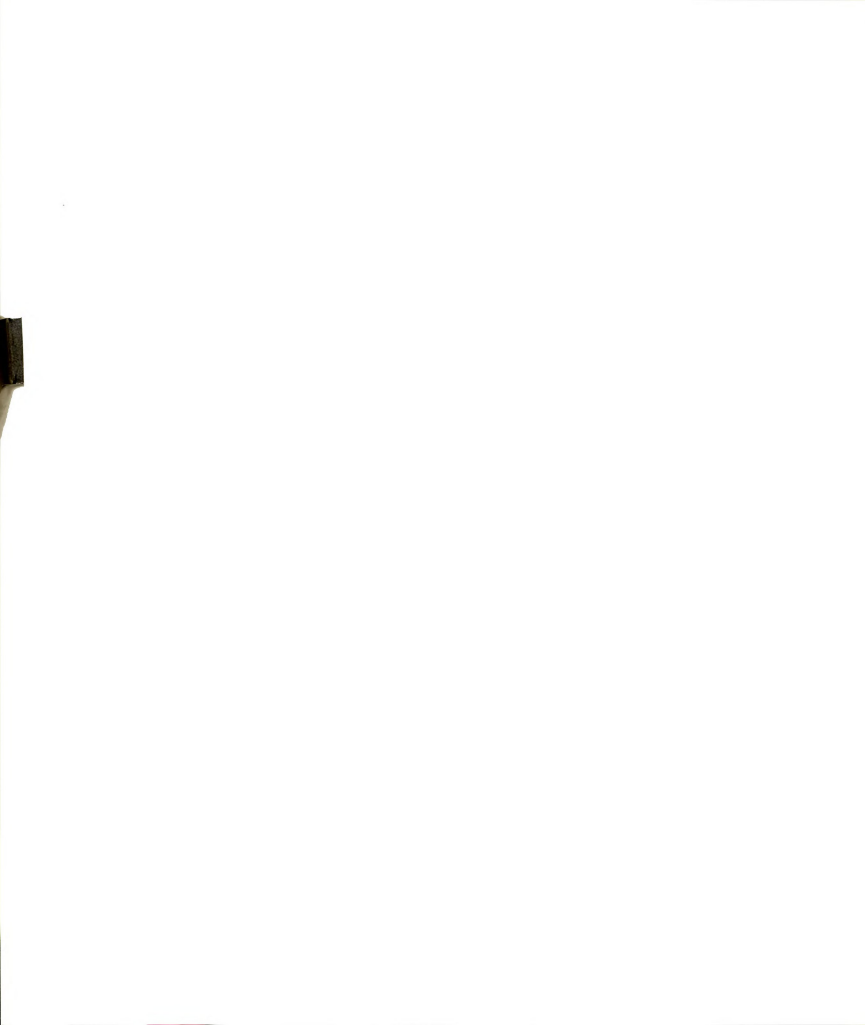
holding tank maintained at 13°C.

Bluegill were transferred to a 1900 liter holding tank and artificial inducement of spawning was begun. On December 19, 1988 the temperature was raised from 13°C to 22°C at a rate of one degree centigrade per hour. Photoperiod was increased from 12 hours light and dark to 18 hours light and 6 hours dark over a period of 14 days.

The bluegill were fed twice daily with commercial trout feed (Zeigler, 5/32 inch pellets). Each week the bluegills were checked for ripeness. Fish were considered ripe when the eggs flowed easily from the genital pore when gentle pressure is applied simultaneously to both sides of the female. Female bluegills began showing signs of ripeness 19 days after the beginning of temperature and photoperiod manipulation.

Each female bluegill that showed signs of ripeness was transferred to a 38 liter aquaria. The aquaria were maintained at 22°C, 0.5 liter/minute flow and equipped with an external standpipe (Garling and Wilson, 1976) to maintain a constant water level in aquaria. The female bluegills were observed twice daily for ripeness.

Female bluegill that had a distended abdomen and swollen genital pore were expected to spawn in three to four days. These female bluegill were given an injection of HCG (Sigma Chemical Co.). HCG was dissolved in distilled water at 1000 I.U./0.1 ml. This solution was injected using a 1



cc syringe and 25 gauge needle (Becton Dickinson).

Injections were intermuscularly at mid-body, just above the lateral line.

Female bluegill were usually ready to spawn two days after injection of HCG. Male bluegill were ready to spawn at 19 days after temperature and photoperiod manipulations. The bluegill were spawned in the laboratory using the same techniques described for naturally ripened bluegill. While these methods were used to complete the pilot study to determine the upper lethal temperature for heat shock treatments of bluegill eggs, the success rate of inducing female bluegill to spawn was approximately 25 percent.

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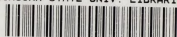


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