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Illinois Trophy Muskellunge Project

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DISCLOSURE

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EXECUTIVE SUMMARY

Recreational fishing is important to the economy of Illinois and its importance is increasing. In 1996, Illinois anglers spent $1.5 billion (ranking 7th in the U.S.) compared to $506 million in 1991 (ranking 13th in the U.S.). Economic output increased from $1.4 billion in 1991 to $3.6 billion in 1996. According to the 1995 Strategic Plan for Illinois Fisheries Resources, muskellunge *Esox masquinongy* and tiger muskellunge, an interspecific cross between female northern pike *Esox lucius* and male muskellunge, account for approximately 2% of the impoundment angling days in 1992. Because of enhanced muskellunge fisheries in the state, it is safe to assume the number of impoundment angling days have increased significantly since 1992. Trophy muskellunge fisheries attract in-state and out-of-state anglers.

Little if any natural recruitment of muskellunge occurs in Illinois. Thus, muskellunge fisheries are produced and sustained by stocking advanced fingerlings. Currently, the IDNR fish hatchery system attempts to stock 16,000 muskellunge per year. Approximately 50% of these fish are males and 50% are females. In muskellunge, the female grows larger, faster, and lives longer than the male (Oehmeke et al. 1958; Becker 1983).

In a study of hundreds of trophy muskellunge (a muskellunge that was mounted) from Canada and the United States, Casselman and Crossman (1986) reported the ratio of females to males was approximately 5.7 to 1. Trophy muskellunge from the United States average 41 inches in total length and range from 2 to 23 years of age.

In most muskellunge waters in Illinois there is a 48-inch size limit. Male muskellunge will probably not reach this length, and few will reach a trophy size in the minds of anglers. Thus, half the production of the IDNR hatchery system and a significant portion of the carrying capacity of the bodies of water in which muskellunge are stocked are occupied by male

muskellunge that will not reach a trophy size. To optimize hatchery rearing space and carrying capacity of muskellunge in receiving waters, it would be desirable to stock all-female advanced fingerlings. Since there is no way to manually separate eggs, larvae, or fingerlings by sex, methods must be developed to produce broodfish capable of producing all-female offspring. This will provide fishery biologists and state and federal entities with a strong tool to manage trophy muskellunge populations. Ultimately, this will benefit anglers.

The purpose of this project is to maximize the efficiency of the Illinois fish hatchery system to produce muskellunge capable of reaching a trophy size to optimize the standing stock of trophy muskellunge in Illinois waters where the fish are stocked. The objective is to develop a muskellunge brood stock that can be used to produce all-female progeny for Illinois stocking programs. The specific objectives include: 1) to determine the muskellunge genetic sex-determination system; 2) to produce masculinized female muskellunge, rear them to sexual maturity and use their sperm to produce all-female off-spring; and 3) to produce intersexed female muskellunge, rear them to sexual maturity, and use their sperm to produce all female off-spring.

The overall strategy used was divided into three components; 1) determining the muskellunge genetic sex-determination system; 2) production of masculinized female muskellunge, rearing them to sexual maturity and using their sperm to produce all-female off-spring; and 3) production of intersexed female muskellunge, rearing them to sexual maturity, and using their sperm to produce all female off-spring. This approach continued throughout the project by necessity given the long period of time required to rear muskellunge to maturity. Specific techniques used in segments 3 and 4 were modified in response to findings from earlier segments.

A previous investigation in muskellunge suggested the presence of a monofactorial (XY or WZ) sex-determining system. We tested this hypothesis using histological and molecular analysis of gynogenetic offspring of muskellunge females. If muskellunge exhibit a monofactorial system, the proportion of female to male gynogens will be 1:0 (XY) or roughly 1:1 skewed in favor of females in proportion to the recombination frequency between the sex-determining locus and the centromere (WZ).

In addition, sex ratios of gynogens should be equal across families unless females exhibit a maternal effect or influence on the offspring. To test for possible maternal effect on sex ratio of gynogens, eggs from eight female muskellunge were activated using UV-irradiated yellow perch sperm and 7000psi pressure shocks were applied to the eggs to produce meiotic diploid gynogens. These gynogen broods were reared in separate indoor tanks to a size when they could be reliably sexed with histological methods. Portions of the eggs obtained each year were used in an investigation designed to evaluate different pressure shock treatments to optimize production of gynogenetic muskellunge.

Samples of 13-20 swim-up gynogenetic fry were taken from these eight broods for microsatellite derived recombination estimates. A portion of gynogenetic individuals were treated with either a dosage of 15mg 17α-methyl-testosterone (MT) or 1000 mg Chrysin per kilogram of feed for 60 days to determine the effects sex reversal on the gonads. A subsample of MT treated gynogenetic muskellunge showed a sex ratio of 17% females, 29% males, 17% immature females, and 37% inter-sex fish (n=24). Chrysin treated fish showed 8% males, 28% females, and 64% inter-sex fish (n=25). This compares to the 89% females to 11% males found in the control fish (n=25).

Remaining gynogenetic swim-up fry from six females were reared as separate broods to approximately 120 mm total length under control conditions. Histological examination of gonads from control gynogens among these six broods indicated 93% were female (n=58). Percentage of females within gynogenetic broods varied from 57%(n=7) to 100% (n=16) indicating sex of gynogens is dependent on maternal origin (modified χ^2 contingency test; $p \leq 0.004$) (Roff and Bentzen 1989).

Six polymorphic microsatellite loci were developed for the muskellunge using an unenriched sub-genomic library. These loci along with a previously developed northern pike locus were used to estimate chromosomal recombination frequencies in gynogenetic muskellunge. Recombination frequencies for the seven microsatellite loci ranged from 0.043 (n=6) to 0.839 (n=2). There was no evidence of a difference in recombination frequency among females. ALFP conducted on five male and six female muskellunge using 58-primer combinations failed to detect a genetic marker specific to either sex. Based on results gathered from this study, it appears unlikely that muskellunge follow either monofactorial XX/XY or WZ/ZZ sex determination systems.

Production of all-female muskellunge using the proposed techniques of gynogenesis and sex reversal is not practical since a monofactorial genetic sex determination is not in operation in muskellunge. Future studies concentrating on identification of mating pairs that produce a preponderance of female offspring may be a viable method for increasing the yield of female muskellunge produced in the state hatchery system. This will require a complex experimental design including multiple cross-matings and progeny tests to identify appropriate male and female broodfish.

Study 101: Hatchery Production of All-Female Muskellunge

Job 101.1: Elucidation of the genetic sex-determination mechanism in muskellunge.

Objective: To identify the genetic sex-determination system in muskellunge as a means to structure other phases of the research

INTRODUCTION

Techniques for reliable mass production of single sex stocks of fishes are dependent on knowledge of the sex determination system in operation. The inheritance of sex in animals may be categorized into two general influences or effects: major and minor sex genetic factors, collectively termed genetic sex determination (GSD), and environmental effects, termed environmental sex determination (ESD) (Bull 1983). ESD systems are rare and have not been identified in any temperate North American freshwater fishes, therefore examination of possible GSD systems is more promising. GSD systems in teleosts, however, are extremely variable ranging from simple monofactorial systems (i.e. XX/XY or WZ/ZZ) to complex polyfactorial systems (i.e. autosomal or WXY) (Kallman 1984; Purdom 1993; Tave 1994).

In a monofactorial GSD system, the sex of an individual is based on the presence or absence of a single genetic element, such as the male determining Y chromosome in a XX/XY system. In a polyfactorial sex determination system, the sex of an individual is determined by the influence of several genetic elements (Bull 1983). Such a mechanism may include one or more loci with the possibility of several allelic states at each. The ability to distinguish male or female sex determining elements can be difficult in polyfactorial systems because of varying degrees of complexity. They also influence the ability to modify the sex ratios of an organism due to the increased complexity of their GSD mechanism.

The currently accepted theoretical paradigm of sex determination systems in most vertebrates is the *a priori* acceptance of a genetically based monofactorial sex determination (i.e., the combinatorialist perspective)(Bull 1983; Purdom 1993). Bull (1983) contends that multiple factor sex determination systems would not remain indefinitely at neutral equilibrium, and random changes in gene frequency would eventually cause GSD systems to revert to a monofactorial heterogametic systems in finite populations. In a monofactorial system, however switches from female heterogamety to a system analogous to male heterogamety can occur when selection favors the spread of autosomal masculinizers (Caubet et al. 2000).

Obtaining an absolute confirmation of the sex determination mechanism in a polyfactorial system using a single analytical technique (e.g., gynogenesis) is improbable. Four commonly used methods for the diagnosis of heterogametic sex in gonorchistic species are cytogenetics (e.g., karyology) (Bull 1983), sex linked markers (e.g., color morphs) (Bull 1983), breeding animals whose sex has been experimentally altered (e.g., sex inversion)(Bull 1983), gynogenesis (Purdom 1993), and androgenesis (Purdom 1993). In muskellunge, prior studies have examined only karyology (Beamish et al. 1971; Davisson 1972; Ráb and Crossman 1994), or sex ratio of gynogenetic progeny (Dabrowski et al. 2000; Rinchard et al. 2002).

The chromosomes of diploid animals occur in duplicate, with one copy from each parent. In some species of fishes (Ebeling and Chen 1970), asymmetry between a single pair of chromosomes in one sex or the other may indicate sex-determining chromosomes. These chromosomes are termed heteromorphic, as they do not match perfectly. An unpaired single chromosome in either sex is also usually representative of a sex chromosome. Studies of Esocid karyotypes to date have revealed a diploid count of 50 homomorphic chromosomes,

with no apparent morphological distinctions among species, or sexes within species (Beamish et al. 1971; Davisson 1972; Ráb and Crossman 1994).

Gynogenesis is an induced form of parthenogenesis where eggs are activated by spermatozoa having denatured nuclear DNA (Thorgaard 1986). Although it does not carry any viable genetic material, the sperm remains motile and may be used to stimulate egg development. To prevent the ova from being haploid, they are subjected to thermal, pressure, or chemical shocks (Pandian and Koteeswaran 1998; Purdom 1969; Stanley 1976; Thorgaard 1986). The shock prevents the loss of the second polar body, resulting in a diploid zygote.

In species with a monofactorial GSD, gynogenesis will produce all female progeny if an XY system is in operation and roughly a 1: 1 male: female if a WZ system is in operation and there is no recombination affecting the sex factors (Stanley 1976). Dabrowski et al. (2000) used gynogenesis along with sex inversion to probe the GSD in muskellunge whose mothers were captured in Clear Fork Reservoir, Ohio. They concluded, based on the fact that males were evident in the gynogenetic progeny, that "...females are heterogametic and possess ZW chromosomes..."(Dabrowski et al. 2000). They showed a ratio of 5:7, males: females, from a sample of 12 gynogenetic progeny produced from between 1 and 5 females. Rinchard et al. (2002) showed 31% female (n=35), 83% female (n=12), and 38% female (n=29) gynogenetic muskellunge in three experimental groups using eggs obtained from between two and five females, and concluded that a WZ/ZZ system was present. Luczynski et al. (1997) suggested female homogameity of the closely related northern pike based on 100% female gynogenetic fingerlings (n=32) generated from a single female. These assumptions, however, do not take into account possible maternal influence on sex ratio, since eggs from only one female or eggs pooled from several female fish were used in each gynogenesis.

Karyology of muskellunge has proven to be ambiguous in determining GSD, and the results from gynogenesis, although suggesting a WZ system, only disprove female homogameity (i.e., XX/XY). Although providing valuable clues, the results of prior studies with muskellunge do not exclude other sex determination systems.

In segments 3 and 4, we chose to expand on results from segments 1 and 2, and studies contemporaneously published by other researchers, by examining maternal specific sex ratio of gynogenetic progeny to determine whether a maternal effect on sex ratio was present. Bull (1983) contended that any of three criteria point to the operation of a polyfactorial sex determination system *vis-à-vis*; 1) high variation in sex ratio among families, 2) paternal and/or maternal effect on familial sex ratio, or 3) sex ratio response to selection. To compliment the prior karyological studies we attempted to identify a sex-linked DNA marker using amplified fragment length polymorphism (AFLP) analysis (Griffiths and Tiwari 1993; Reamon-Büttner et al. 1998; Griffiths and Orr 1999; Griffiths et al. 2000; Reamon-Büttner and Jung 2000). If a monofactorial GSD was present AFLP analysis has a high probability of identifying a sex-linked DNA marker since in monofactorial GSD systems the decisive gender determinant is usually conserved (Purdom 1993; Griffiths and Orr 1999).

Results from segment 2 of this investigation showed a sex ratio of approximately 8 females: 1 male (n=28) in a sample of gynogenetic muskellunge taken in fall of 2000. This skewed ratio is possible if, in the WZ system, recombination occurs between chromosomes during the prophase of meiosis I (Avtalion and Don 1990). If the sex determination element on the sex chromosome behaves as a single locus, then three potential genotypes of gynogenetic progeny are possible: WW, WZ, and ZZ, with the ratios of each dependent upon the rate of crossing over. In this 9:1 female: male phenotypic sex ratio, genotypic proportions of

WW:WZ:ZZ individuals should be approximately 1:8:1 as both homozygous states should be equally frequent and the frequency of heterozygotes is directly related to the chromosomal recombination rate (Thompson 1983). This indicates a recombination frequency of approximately 0.80 for a single locus on a pair of sex chromosomes.

This estimate of recombination frequency was derived from a pooled sample of gynogenetic offspring. It may be possible that differential variability in recombination occurs between female muskellunge, resulting in skewed sex ratios between families of gynogenetic progeny. Recombination frequencies can be calculated from heterozygosity of meiotic gynogenetic progeny, since heterozygotes among gynogenetic offspring of known heterozygous females must have arisen by a recombination event (Purdom et al. 1976; Thompson 1983). Thompson and Scott (1984) showed variation in heterozygotes among gynogenetic broods in rainbow trout *Salmo gairdneri* using three allozyme loci. To test the possibility that any skewed sex ratios among gynogenetic broods were related to maternal influence or the result of individual variation in recombination frequency, microsatellite polymorphisms were used to estimate recombination frequencies of each female.

METHODS

1999 (Segment 1)

Female broodfish were collected from two Illinois lakes, Spring Lake during March 1999, and Kinkaid Lake during April 1999. The nuclear material of northern pike sperm was destroyed following the procedure outlined in Lin and Dabrowski (1996). The semen was extended approximately 9:1 using a semen extender (0.75% NaCl and 0.6% KCl)(Varadaraj 1990), placed under a short wave (254 nm) UV light for a period necessary to provide a level of UV exposure $\geq$ 2,000 J/m^2. The irradiated sperm was then used to activate the ova using

standard culture methods for muskellunge (Westers and Stickney 1993). After 20 minutes the ova were temperature shocked at 30°C for 8 minutes in order to produce diploid eggs. These eggs were then incubated. The putative gynogens were to be reared until they could be sexed. We intended to use histological methods to identify sexes prior to sexual maturation (Lin et al. 1997).

We also investigated producing gynogens using pressure shocks following a procedure used to produce triploid hybrid *Lepomis* by Wills et al. (1994). Four different shock regimes were attempted varying pressure, post-fertilization time of shock initiation, and shock duration.

We anticipated the sex ratio in gynogens to be one of the following: 1) 100% female, indicating an XY system in muskellunge; 2) roughly 1:1 males: females, indicating a WZ system; or 3) 100% males, indicating a WZ system, but WW individuals are not viable.

2000 (Segment 2)

Female broodfish were collected from two Illinois lakes, Spring Lake during early March 2000, and Kinkaid Lake during late March 2000. Sperm from walleye was used to fertilize ova taken from the Spring Lake females, and yellow perch sperm was used for the ova taken from Kinkaid Lake females. The nuclear material of the sperm in both cases was destroyed following the procedure outlined in Lin and Dabrowski (1996). The semen was extended approximately 9:1 using a semen extender (0.75% NaCl and 0.6% KCl)(Varadaraj 1990), placed under a short wave (254 nm) UV light for a period necessary to provide a level of UV exposure of 648 J/m^2 for yellow perch sperm and either 648 J/m^2 or 2,160 J/m^2 for walleye sperm. The irradiated sperm was then used to activate the ova using standard culture methods for muskellunge (Westers and Stickney 1993). Two different shock procedures were used in order to produce diploid eggs, temperature shocks and hydrostatic pressure shocks. The

temperature shock used was the same as in segment 1, after 20 minutes the ova were temperature shocked at 30°C for 8 minutes. Several hydrostatic pressure shocks were used with the time of shock initiation after fertilization being varied. This is similar to the procedure used to produce triploid hybrid *Lepomis* by Wills et al. (1994). Pressure was constant at 7000 psi for all treatments and duration of all of the shocks was 3 min. The eggs were then incubated and hatched. The resulting gynogenic muskellunge were reared on pelleted feed followed by fathead minnows until they were large enough to be sexed reliably. Histological methods were used to identify sexes prior to sexual maturation (Lin et al. 1997).

We also investigated the application of alternative egg activation techniques that could eliminate the need for sperm (i.e., true parthenogenesis). Samples of eggs from three females were separated into seven treatment and control batches (one set of treatments and controls from each female) to determine the effectiveness of two activator solutions for producing parthenogens. The egg exposure treatments were: 1) muskie sperm, and water (fertilized control); 2) water, and no sperm (unshocked negative control); 3) water, no sperm, and a pressure shock (shocked negative control); 4) 10 mM NH_4Cl for 30 sec, water rinse, and pressure shock (Ammonium 30 sec); 5) 10 mM NH_4Cl for 1 min, water rinse, and pressure shock (Ammonium 1 min); 6) 1 M Urea for 30 sec, water rinse, and pressure shock (Urea 30 sec); and 7) 1 M Urea for 1 min, water rinse, and pressure shock (Urea 1 min). All of the pressure shocks were conducted at 7000 psi for 3 min duration begun 20 min after exposure to the treatment. The eggs were incubated at 54° F (12° C) until hatching occurred. Development was observed periodically to determine if any abnormalities occurred. Dead eggs were removed from the containers daily so that they would not hinder the development of the other eggs.

Approximately 100 of the gynogenic muskellunge produced in year 2000 were separated to be reared for the sex control mechanism determination. When the fished reached a mean size of 8.2 inches (SD=1.6) a subsample of 28 fish were sacrificed for histological determination of their sex.

Again, we anticipated the sex ratio in gynogens to be one of the following: 1) 100% female, indicating an XY system in muskellunge; 2) roughly 1:1 males: females, indicating a WZ system; or 3) 100% males, indicating a WZ system, but WW individuals are not viable.

2001 (Segment 3)

Female broodfish were collected from two Illinois lakes, Spring Lake during early March 2001, and Kinkaid Lake during late March 2001. Yellow perch spermatozoa were used to activate ova taken from the muskellunge females. The nuclear material of the sperm was destroyed following the procedure outlined in Lin and Dabrowski (1996). The semen was extended approximately 9:1 using a semen extender (0.75% NaCl and 0.6% KCl)(Varadaraj 1990), placed under a short wave (254 nm) UV light for a period necessary to provide a level of UV exposure of 648 J/m^2. The irradiated sperm was then used to activate the ova using standard culture methods for muskellunge (Westers and Stickney 1993).

Hydrostatic pressure treatments were used to prevent second polar body expulsion to produce diploid eggs. After activation of the ova, several hydrostatic pressure shocks were used with varying time of shock initiations and durations. This is similar to the procedure used to produce triploid hybrid *Lepomis* spp. by Wills et al. (1994). Pressure was held constant at 7000 psi for all treatments and duration of all of the shocks was 3 min. The eggs were then incubated in Macdonald jars and hatched. The resulting gynogenic muskellunge were reared on pelleted feed followed by fathead minnows until they were large enough to be sexed

reliably. Histological methods were to be used to identify sexes prior to sexual maturation (Lin et al. 1997).

Sex-linked DNA Marker

Five adult male and six adult female muskellunge were captured from Spring Lake in Illinois by trap netting during March of 2001. Pelvic fin clips were only taken from male fish confirmed by milt expression and female fish confirmed by egg expression. These tissue samples were preserved in 1.5ml Eppendorf® tubes containing 95% ethanol. Genomic DNA from male and female fin clips was isolated using a Qiagen DNeasy Tissue Preparation Kit (Qiagen, Cat. No. 69506) and stored at –20°C.

AFLP analysis was employed to determine polymorphism between male and female muskellunge genomic DNA. Technical procedure for running AFLP can be taken from Vos et al. (1995), but we used GibcoBRL AFLP Analysis System I (Life Technologies, Cat. No. 10544-013). Genomic DNA was digested with two restriction enzymes (*Eco*RI and *Mse*I) in 10mM Tris (pH 7.5), 10mM MgAc, and 50mM KAc. Ligation of 100ng of two double stranded adapters to the *Eco*RI and *Mse*I sites was performed in adapter ligation solution with one unit of T_4 DNA ligase at 20°C for 2 hours. Ligation reactions were diluted 1:10 in TE buffer (10mM TrisCl pH 8.0, 0.1mM EDTA). Preamplification PCR was performed with 5µl of diluted template DNA, 40µl pre-amp primer mix containing dNTP's and *Eco*RI and *Mse*I primers complementary to the adapters with one additional preselective nucleotide at the 3'end, 1 unit *Taq* polymerase, and 5µl 10X PCR buffer (200mM TrisCl pH 8.3, 15mM $MgCl_2$, 500mM KCl). Reactions were placed in a Hybaid Omn-E Thermal Cycler (Hybaid Unlimited Perkin-Elmer Corporation) with parameters of 20 cycles at 94°C denaturing for 30 seconds, 56°C annealing for 60 seconds, and 72°C extension for 60 seconds. Pre-amp reactions were

diluted 1:50 in TE buffer and selective PCR amplifications were conducted using *Mse*I and *Eco*RI primer combinations with 3 additional selective nucleotides at the 3' end as follows. *Eco*RI primers were end labeled using [γ-^{32}P]ATP and T_4 polynucleotide kinase. Twenty µl PCR reactions contained 5µl of diluted pre-amp DNA as template, *Mse*I and labeled *Eco*RI primers, 20mM Tris (pH 8.4), 1.5mM $MgCl_2$, 50mM KCl with dNTPs, and two units *Taq* polymerase. Selective amplifications were performed in a Hybaid Omn-E thermal cycler with the following thermal parameters: 94°C denaturing for 60 seconds, 1 cycle beginning at 65°C annealing step down 1°C per cycle to 56°C annealing for 60 seconds each, and 72°C extension for 60 seconds; 23 at cycles 94°C denaturing for 30 seconds, 56°C annealing for 30 seconds, and 72°C extension for 60 seconds. An equal volume of loading dye (98% formamide, 10mM EDTA, xylene cyanol, and bromophenol blue) was added to each reaction and reactions were incubated at 95°C for ten minutes, then placed on ice before loading. Three µl of each mixture was loaded onto a 0.4mm 6% denaturing polyacrylamide gel and electrophoresed at 60 volts until the second dye (xylene cyanol) had run 2/3 of the way down the gel (approximately 2-3 hours). Gels were dried to a sheet of 3mm Chr 46x57cm filter paper (Whatman International Ltd., CAT 3030-917) using a Model 583 Bio-Rad Gel Dryer (Bio-Rad Laboratories) and RVT 100 Savant refrigerated vapor trap. Dried gels were exposed to 35x43cm Kodak Biomax MS double emulsion Scientific Imaging Film (CAT 143 5726) overnight. Films were developed and fixed using Kodak GBX Developer and Fixer (CAT 190-1859).

2002 (Segment 4)

Nine female muskellunge were collected from two Illinois lakes, Spring Lake during early March 2002, and Kinkaid Lake during late March 2002. Denatured yellow perch spermatozoa were used to activate ova taken from the muskellunge females (Dabrowski et al.

2000). The semen was extended approximately 9:1 using a semen extender (0.75% NaCl and 0.6% KCl) (Varadaraj 1990), placed under a short wave (254 nm) UV light for a period necessary to provide a level of UV exposure of 648 J/m^2 (Malison et al. 1996). The irradiated sperm was then used to activate the ova using standard culture methods for muskellunge (Westers and Stickney 1993).

After activation, hydrostatic pressure treatments were used to prevent second polar body expulsion to produce diploid gynogenetic embryos (Garcia-Abiado et al. 2001). The embryos from each female (brood) were then incubated separately in Macdonald jars and hatched. Sac fry from each brood were reared in separate nylon mesh baskets until swim-up. After yolk-sac absorption and the onset of exogenous feeding, 13-20 larval fish were sampled from 8 of the 9 females (684A, 5C3A, 65720, C1827, 70822, 63E77, 47F79, and 5B0F) and stored in 1.5 ml Eppendorf® tubes containing 95% ethanol for microsatellite analysis (below). By this stage of development, all of the fish sampled were diploid gynogens and not haploids since haploid fish fail to swim-up and take feed (Dabrowski et al. 2000). Gynogenetic families were kept separate throughout the rearing process.

Sex Ratios of Gynogenetic Broods

The remaining gynogenetic progeny from six of the eight female muskellunge (684A, 5C3A, 70822, 63E77, 47F79, and 11809) were reared on pelleted feed in separate 50 gallon circular tanks, all with a common water supply, according to standard culture procedures until they were large enough to be sexed reliably (Piper et al. 1982; Harvey 1986; Jorgensen 1986; Klingbiel 1986; Westers and Stickney 1993). Histological methods were used to identify sexes prior to sexual maturation (mean total length=122.5 mm; SD=10.6) (Lin et al. 1997). Between 5 and 16 survivors from each brood were preserved in 10% neutral buffered formalin. Histological examination using routine hematoylin/eosin staining of 4μm gonad cross sections

was conducted to determine phenotypic sex. Sections of the gonad were examined with light microscopy at 100X to 400X for the presence ovaries or testes (Lin et al. 1997). The ratio of male: female gynogens was calculated for each of the broods. A modified χ^2 contingency test was calculated to test the hypothesis that sex ratio was independent of mother (Roff and Bentzen 1989).

Microsatellite Isolation

A pelvic fin clip was obtained from a single muskellunge captured from Spring Lake and preserved in a 1.5ml Eppendorf tube containing 95% ethanol. Genomic DNA was isolated using a Qiagen DNeasy Tissue Preparation Kit (Qiagen) and stored at –20°C. The procedure for isolation of microsatellites from a sub-genomic library followed that of Heist and Gold (2000). Isolated muskellunge genomic DNA from the fin clip was digested with *Mbo*I restriction enzyme. Resulting fragments were separated using a 1% TAE agarose gel electrophoresis and fragments spanning 300-800 base pairs were excised from the gel using a sterile scalpel. DNA from gel slices was extracted using QIAquick Gel Extraction Kit (Qiagen), ligated into pUC 18 cloning vector (Amersham Pharmacia Biotech), and used to transform DH5α competent cells (Invitrogen Life Technologies). Approximately 1200 individual colonies were transferred to a 0.45-micron MAGNA nylon transfer membrane (Osmonics) and probed with ^{32}P radiolabelled $(GT)_{10}$ and $(GA)_{10}$ oligo sequences.

Thirty-three positive clones were isolated using Wizard Plus Minipreps DNA Purification Systems (Promega) and manually sequenced by the dideoxynucleotide chain termination method using M13 primers and *fmol* DNA Cycle Sequencing System (Promega). Sequences flanking the microsatellite core repeat regions were used to design forward and reverse PCR primers with MacVector 6.5.3 software (Oxford Molecular Group Ltd. 1998). Primers were screened using 24 muskellunge samples and the cloned fragment as a size

standard. Forward primers for each microsatellite locus were radiolabeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase as follows: 0.6µl forward primer (100pmol/µl), 1.55µl dH20, 0.5µl 10X PNK buffer (Epicentre), 2.1µl [γ-^{32}P]ATP, and 0.25µl kinase (1U/µl). The kinase reaction was incubated at 37°C for 30 minutes and 95°C for 10 minutes. PCR reactions (10µl) contained 1-15ng DNA template, 1X PCR buffer (50mM KCl, 10mM Tris-HCl pH 9.0, and 0.1% Triton X-100), 200mM each dNTP, 2mM $MgCl_2$, 0.2 units *Taq* polymerase, and 10mM reverse primer, 0.66µl labeled forward primer. Amplifications were initially conducted in an Eppendorf Master Cycler Gradient at annealing temperatures ranging from 50°C to 64°C to identify optimal annealing temperatures. Thermal parameters for the amplification consisted of a two-minute denaturing step at 94°C, followed by 25 cycles of 94°C for 30 seconds, 60-64°C for 30 seconds, and 72°C for 30 seconds. PCR products were separated with a 6% denaturing polyacrylamide gel electrophoresis.

Microsatellite Recombination Frequencies

Fin clips from 8 of the 9 female muskellunge used to create gynogens were preserved in 1.5ml Eppendorf tubes containing 95% ethanol. DNA from fin clips of each female and 13-20 of her respective larval gynogenetic progeny were isolated using the aforementioned procedure. Six microsatellite loci developed for northern pike (*Elu*252, *Elu*2, *Elu*6, *Elu*51, *Elu*19, and *Elu*276) were used to examine heterozygosity of each female (maternal) DNA and her respective gynogenetic progeny (Miller and Kapuscinski 1996, 1997; Hansen et al. 1999). Additionally, 6 microsatellite loci isolated for muskellunge (previous section) were also used. Microsatellite primer sequences were obtained from Intergrated DNA Technologies, Inc. PCR reactions and forward primer end labeling were conducted as previously described. Initial PCR amplification was carried out in an Eppendorf Master Cycler at annealing temperatures ranging from 50°C to 64°C to identify optimal annealing temperatures. Once optimal

annealing temperatures were established, each amplification consisted of a two-minute denaturation step at 94°C, 25 cycles of 94°C for 30 seconds, 60-64°C for 30 seconds, and 72°C for 30 seconds. PCR products from each sample were separated using a denaturing 6% polyacrylamide gel electrophoresis and visualized with autoradiography using cloned fragments as size standards. Heterozygosities of gynogenetic progeny in broods derived from heterozygous mothers were used to calculate the ratio of non-recombinants to recombinants at each locus. A modified chi-square contingency test was calculated to test the hypothesis that frequency of recombination was independent of mother (Roff and Bentzen 1989).

RESULTS AND DISCUSSION

1999 (Segment 1) RESULTS AND DISCUSSION

A total of 1,835 ml of muskellunge eggs were obtained from 15 female muskellunge with the aid of personnel at the Jake Wolf State Fish Hatchery. Gynogenesis induction techniques were applied to these eggs producing a total of 365 swim-up fry (Table 1). The viability of these eggs at the eyed stage ranged from 0.7 % to 64.4 %. The percentage of swim-up fry produced ranged from 0.0 % to 2.1%. When these grew to fingerling size a sample was taken for protein electrophoretic analysis to test whether they were pure muskellunge, and thus gynogens. All of the survivors were determined to be muskellunge X northern pike hybrids.

During growout of these fish, though, it was noticed that there were two different size classes. This difference in sizes was not particularly great given the small size of the fish. The implications of the difference was not realized until, over the course of a few hours, the larger individuals cannibalized all of the smaller individuals. It is possible that the smaller individuals were pure muskelluge (gynogens) given that the faster growing individuals were all

hybrids. Therefore, in segment 2 an alternative species, such as yellow perch *Perca flavescens* or Walleye *Stizostedion vitreum*, was used as the sperm donor for producing gynogens. We also investigated the application of alternative egg activation techniques that could eliminate the need for sperm.

Aided by IDNR biologist, Shawn Hirst, 153 muskellunge were captured from Kinkaid Lake in Southern Illinois. Of these, only two had ripe eggs, one produced 100 ml of eggs, the other produced 1000 ml (Table 2). The eggs from the former produced 43 swim-up fry, and eggs from the later failed to develop. The few fish produced from the Kinkaid broodfish succumbed to disease during growout, six moribund fish were retained for protein electrophoresis, all were determined to be pure muskellunge and thus gynogens. A hydrostatic pressure shock of 7000 psi applied at 15 minutes post-fertilization for a duration of 3 minutes was used to produce these individuals. Thus, we have demonstrated the ability to produce gynogens. Since gynogens show poor survival due to inbreeding, obtaining more eggs from more spawns will be critical to the success of this project in the future.

2000 (Segment 2) RESULTS AND DISCUSSION

A total of 3,040 ml of muskellunge eggs were obtained from fish handled by the personnel at the Jake Wolf State Fish Hatchery and the IDNR biologists Wayne Herndon and Rob Hilsabeck. Gynogenesis induction techniques were applied to these eggs producing a total of 828 viable swim-up fry (Table 3). Aided by IDNR biologist, Shawn Hirst, seven ripe female muskellunge were captured from Kinkaid Lake in Southern Illinois that produced 4,310 ml of eggs. These eggs produced 1,509 swim-up fry (Table 4). The eggs from females taken by Jake Wolf Fish Hatchery were fertilized with sperm taken from walleye; those from Lake Kinkaid were fertilized with sperm from yellow perch. Since either walleye or yellow perch

were used as sperm donors to produce these fish, the muskellunge produced are unambiguous gynogens. Incomplete denaturing of the sperm nucleus would have resulted in a interfamilial hybrid that is non-viable (Dabrowski et al. 2000). A total of 556 three inch to 3 ¾ inch gynogenic muskellunge fingerlings were produced, 105 from Jake Wolf spawning, and 451 from Kinkaid Lake spawning. One hundred and six fish were reserved for the genetic sex-determination mechanism evaluation, and 450 were used for Job 101.2 (Segment 2) (below).

Histological analysis of the subsample of gynogens sacrificed on November 3, 2000 showed a sex ratio of 89% females to 11% males. This confirms the results of Dabrowski et al. (2000) that an XY system does not appear to be the genetic sex-determination mechanism in the muskellunge. Instead it appears that a WZ may be in operation. Note, though, that we did not show a 1:1 ratio of males:females as expected with the WZ system. The likely causes of this are that crossing over is occurring during the first reduction division of meiosis I of gametogenesis in the parental female, or there is some other genetic sex-determination mechanism is at work. Crossing-over would result in ova that contain either WW or WZ chromosomes rather than the expected WW chromosome set. The proportions of WW:WZ:ZZ individuals should be approximately 1:8:1, based on our percentages assuming the WW bearing individuals are viable.

In our initial trials of parthenogenesis none of the experimental treatments resulted in viable embryos. However, embryos were produced in the fertilized control group, showing that the eggs were capable of development. Microscopic examination of the eggs during development showed that activation seemed to be occurring in all of the groups including both of the negative controls. However, development in all but the fertilized controls eventually became abnormal. The abnormal embryos subsequently died. So, urea, ammonium chloride,

and even plain water are capable of activating eggs. However, they are not capable of supporting competent development, even when a pressure shock that is known to produce gynogens is applied to them.

2001 (Segment 3) RESULTS AND DISCUSSION

Gynogenesis induction techniques were applied producing a total of 1,724 viable swim-up fry. Since yellow perch were used as sperm donors to produce these fish, the muskellunge produced were unambiguous gynogens. Incomplete denaturing of the sperm nucleus would have resulted in an interfamilial hybrid that is non-viable (Dabrowski et al. 2000). A total of 462 three inch to 3 ¾ inch gynogenic muskellunge fingerlings were produced. Of the gynogens, we reserved 184 fry for the genetic sex-determination mechanism evaluation, and we used 278 fry for Job (Segment 3)101.2.

During the last segment, histological analysis of the sub-sample of gynogens sacrificed on November 3, 2000 indicated a female percentage of 89% and 11% for males. This confirms the results of Dabrowski et al. (2000) that an XY system does not appear to be the genetic sex-determination mechanism in the muskellunge. Instead, it appears a WZ system may operate in muskellunge. Though, we did not show a 1:1 ratio of males:females as expected with the WZ system. The likely causes of this are crossing-over is occurring during the first reduction division of meiosis I of gametogenesis in the parental female, or there is some other genetic sex-determination mechanism at work. Crossing-over would result in ova that contain a WZ complement of chromosomes as well as ova containing the expected WW or ZZ chromosome sets.

Sex-linked Marker

Using 5 male and 6 female muskellunge DNA samples, total of 2834 DNA fragments were scored using 58 primer combinations, based on restriction enzymes *Mse*I and *Eco*RI with

three base pair extensions each. Of the 2834 DNA fragments scored, 209 of them were variable in at least 1 of the 11 individuals tested (Table 6). The proportion of variable to fixed fragments for all 58-primer combinations was 0.0735. Each DNA fragment was assumed to be a discrete locus, and individuals were scored based on the presence or absence of each fragment on the gel. Average heterozygosity observed (H_o=0.0286) was estimated using the Hardy-Weinberg equation and allele frequencies across all loci, including both fixed and variable fragments. None of the variable fragments examined resulted in a marker exclusive to either male or female sex.

2002 (Segment 4) RESULTS

Sex Ratios of Gynogenetic Broods

Histological examination yielded clear distinction between developing males and females among the six broods of gynogenetic muskellunge examined. No hermaphrodites or other developmentally aberrant individuals were detected. Overall, 93.0% of the gynogenetic progeny among the six broods were female (n=58) (Table 5). The sex ratios among families varied from 57% to 100% females with the majority of broods (67%) exhibiting 100% females. Roff and Bentzen's (1989) modified chi-square contingency test of the hypothesis that sex ratio was independent of the maternal origin (i.e., brood) of the gynogenetic progeny was not supported ($p \leq 0.004$). No obvious trends could be detected with regard to influence of shock treatments on sex ratio of gynogenetic progeny.

Microsatellite Recombination Frequencies

Six of the nine muskellunge microsatellite loci were polymorphic in the female muskellunge sampled and all six of these loci had heterozygous mothers, ranging in number from two to eight (Table 7). Only one of six northern pike microsatellite loci amplified polymorphic products in muskellunge (n=8). This locus, *Elu*51, yielded two alleles, with two

females being heterozygous. Microsatellite amplifications were performed on between 13 and 20 gynogens from each heterozygous female to identify the loss of heterozygosity for each gynogenesis (Table 8). Average heterozygosity at a locus (i.e., recombination frequency) ranged from 0.043 to 0.839 and the average overall observed heterozygosity for all gynogen broods across all loci was 0.576 (SD=0.237). Roff and Bentzen's (1989) modified chi-square contingency test of the hypothesis that recombination frequency was independent of the maternal origin (i.e., brood) of the gynogenetic progeny was supported at all seven loci after a sequential Bonferroni adjustment of alpha (Cooper 1968; Rice 1989) (Table 8).

2002 (Segment 4) DISCUSSION

Three findings emerged from this investigation: (1) AFLP did not resolve a genetic marker exclusive to either the male or female sex; (2) the sex ratio results observed among gynogenetic broods reflect two genetically different maternal genotypes, the first genotype giving rise to both gynogenetic males and females, and the second genotype giving rise to females only; (3) sex ratio results were not supported by differential recombination frequencies between females. These results do not support the possibility of a monofactorial WZ/ZZ sex-determination system as suggested by Dabrowski et al. (2000) and Rinchard et al. (2002).

Although explaining the possibility of complex polyfactorial GSD systems, Bull (1983) states that the occasional exception to an otherwise simple mechanism for the inheritance of sex does not does not warrant abandoning the overriding theme of simplicity. Bull classifies mechanisms as male and female heterogamety even if they are known to produce occasional exceptions. Purdom (1993) supports this modern tendency toward the theme of simpler monofactorial systems as well.

Monofactorial GSD systems are well documented in both mammals (XX/XY) and birds (WZ/ZZ) with rare exceptions (Graves and Shetty 2001). All members of these two taxa, however, have heteromorphic chromosomes, with structurally derived and distinguishable sex chromosomes (Bull 1983). Identification of heteromorphic chromosomes in fishes, however, is usually not the case. Of the 810 species of osteichthyan fishes listed by Sola et al. (1991), only 28 have confirmed heterogamety through karyotyping.

To assume the operation of monofactorial sex determination systems in fishes may not be valid, especially when considering the diversity of this taxon (Gordon 1946; Ebeling and Chen 1970). It is also probable that advanced systems, such as heteromorphic XX/XY chromosomes, originated from genetically homologous sex chromosomes (Charlesworth 1991). This would indicate a degree of sex chromosome differentiation that accompanies a change in recombination patterns following the establishment of a major sex determination element (Carrasco et al. 1999). The conserved sex determination element in the heterogametic sex should have been identified by AFLP as a locus (loci) on the sex chromosome not possessed by the homogametic sex.

Possible explanations for this are either the non-recombining locus (loci), which determines sex cannot be identified by the resolution offered with AFLP, or there is a more complex sex-determination mechanism than a monofactorial. The WZ/ZZ sex determination system in muskellunge is supported by the presence of both the male and female sex within gynogen yields (Dabrowski et al. 2000). This proves female heterogamety, but does not rule out more complex sex determination systems (Bull 1983; Avtalion and Don 1990; Mair et al. 1991).

Recombination of sex chromosomes has been previously suggested to explain non-Mendelian sex segregations in interspecific crosses of tilapias (Wohlfarth and Wedekind 1991). In a WZ/ZZ system, obligatory recombination between homologous sex chromosomes would yield 100% WZ female offspring. Complete absence of recombination between homologous sex chromosomes would yield 50% WW female offspring and 50% ZZ male offspring, and equivalent rates of recombination between homologous sex chromosomes would yield both males and females, with a skew towards femaleness in direct proportion to the recombination frequency of the locus. In any of these three cases, the sex ratios of gynogenetic progeny generated through these models of recombination should be equal across all broods, as statistically significant variable recombination frequencies were not exhibited by females in the microsatellite portion of this investigation. Heterozygosity of gynogenetic progeny does suggest differential recombination frequencies between loci, as reported by Thorgaard (1986) and loci that remain at a high proportion of heterozygotes suggest distal position of the locus in relation to the centromere.

Characteristically, fishes possess great diversity with respect to sex determination (Tombka and Avtalion 1993). A group of fishes with a notoriously complex history of sex determination investigations are the tilapias. Avtalion and Don (1990) discovered two genetically different maternal genotypes in F_2 gynogenetic tilipia *Oreochromis aureus*. One female type would give rise to only female gynogenetic progeny and the second female type would give rise to both male and female gynogenetic progeny suggesting a WZ/ZZ system. None of the F_1 gynogenetic tilapia however produced all female progeny, indicating the absence of WW individuals after the first gynogenesis. They concluded that a simple sex-

determination mechanism based on heterogamety of females was not the case as males were persistently present among F_1, F_2, and F_3 gynogenetic offspring.

Mair et al. (1991) reported aberrant sex ratios of progeny tests using sex inverted *O. aureus* individuals suggesting an autosomal recessive element epistatic to a major GSD. Sex ratios of triploid and gynogenetic offspring support recombination of sex element resulting in a skewed sex ratio in favor of females from the expected 1 male: 1 female. These authors concluded *O. aureus* has a multifactorial GSD with an underlying WZ/ZZ system.

To explain observed sex ratios of *O. niloticus* meiotic and mitotic gynogenetic progeny and crosses of these progeny with normal individuals, Müller-Belecke and Hörstgen-Schwark (1995) suggested the occurrence of two or more minor sex determining factors in which are able to override the XX/XY mechanism.

Scott et al. (1989) reported intercrosses among hormone sex-reversed *O. niloticus* with normal males and females resulted in progeny with aberrant sex ratios suggesting an XX/XY system with a minor sex ratio modifying factor. Sex reversed females were crossed with normal females resulting in predominantly female offspring.

Wohlfarth and Wedekind (1991) review investigations of interspecific hybrids, intraspecific hybrids, chromosome manipulations, and sex inversions of members of the genus *Oreochromis*. Based on data from these investigations, they concluded that the mechanism of sex determination in tilapias is analogous to that of the platyfish (WXY) described by Kallman (1984). They also found selected males that repeatedly generated predominantly male offspring regardless of the female mate, but stated that the chance of producing super males to generate all male broods was slim to none. Scott et al. (1989) obtained exceptional male *O. niloticus* through meiotic gynogenesis that sired predominantly male progeny as well.

Mair et al. (1991) observed greatly varied sex ratios in interspecific hybrid crosses between *O. aureus*, *O. niloticus*, *O. spilurus*, and *O. mossambicus*. These authors concluded that due to the considerable complexity of interspecific sex determination, few valid inferences could be made regarding sex determination from hybrid sex ratio data.

Trombka and Avtalion (1993) review four approaches that have been used to identify sex determination systems in *Oreochromis*: (1) inter- and intraspecific crosses, back crosses and crosses between different hybrids of different generations, (2) sex inversion of fry to females or males by hormonal treatment, (3) chromosome manipulations leading to polyploidy and gynogenesis, and (4) karyotyping and differential staining of the tilapia genome. The bulk of existing inter- and intraspecific hybrid data point to homogametic and heterogametic genders within each species and a dual system of sex chromosomes in tilapias, with unexpected sex ratios indicating other factors are involved in sex determination. These authors conclude polygenenetic sex determination cannot be ruled out and sex determination in this genus is still unresolved.

These discoveries in tilapias have shown that monofactorial sex determination may not apply to all fishes. Although not providing clear evidence for an exact model of sex determination, the results of this investigation support the operation of a polyfactorial GSD system in muskellunge. It appears unlikely that muskellunge follow either an XX/XY or a WZ/ZZ sex determination system, which indicates that production of WW super females as suggested by Garcia-Abiado et al. (2001) might not be possible. Single analytical techniques, such as gynogenesis, may not provide all of the clues required to identify possible sex determination mechanisms and assumptions based on monofactorial GSD systems may not always be appropriate for all fishes. Future breeding programs, however, may identify crosses

between muskellunge with the propensity to produce predominantly female offspring, as maternal influence has been shown to effect sex ratio of gynogenetic offspring.

Production of all-female muskellunge using the proposed techniques of gynogenesis and sex reversal is not practical since a monofactorial genetic sex determination is not in operation in muskellunge. Future studies concentrating on identification of mating pairs that produce a preponderance of female offspring may be a viable method for increasing the yield of female muskellunge produced in the state hatchery system. This will require a complex experimental design including multiple cross-matings and progeny tests to identify appropriate male and female broodfish.

Job 101.2: Production of masculinized female muskellunge broodfish.

Objectives: To maximize the production of gynogenic female muskellunge, masculinize them, hold them to sexual maturity and use their semen to produce all-female progeny.

INTRODUCTION

If muskellunge had an XY system (i.e., females are homogametic or XX), then there are two ways to produce monosex female populations, other than hormonal sex reversal. Brood fish can be produced that generate sperm containing only the X chromosome. The first step is to produce all female muskellunge by the process known as gynogenesis. Gynogens are produced by irradiating the sperm of normal male muskellunge with UV light which destroys the nuclear DNA. The sperm remains intact and is motile; however it does not carry any viable genetic material. The irradiated sperm is then used to stimulate egg development. To prevent the ova from being haploid, they are subjected to either a thermal or pressure shock. The shock prevents the loss of the second polar body, resulting in a diploid cell (Stanley 1976).

Gynogenesis has been successfully accomplished on many species of fish including muskellunge (Lin and Dabrowski 1996); however, the genetic sex control mechanism of muskellunge is not understood. If all of the fish produced in gynogenesis were homogametic females (XX) for a XX/XY system, it would have been possible to stop with this first step. However, since gynogens tend to have low survival and they are highly inbred, this limits their usefulness for stocking (Dunham 1990). This problem has been overcome in trout by feeding young genotypic female gynogens food that contains a masculinizing hormone such as methyltestosterone (MT) so they phenotypically produce sperm. The sperm from these sex reversed brood fish bear only X chromosomes, and they can then be used to produce all-female fingerlings for stocking simply by crossing them with normal females. These out-crossed fingerlings are essentially not inbred and should have a normal mortality rate. Also, they have never been exposed to exogenous hormones. Had all female lines bene developed through gynogenesis alone, then some of the female progeny could have been masculinized during each production year to serve as future brood stock. This means gynogenesis need have only be conducted once, as long as the female lines are perpetuated in the hatchery system.

Alternatively, if the sex determination system had proven to be WZ and gynogens are treated with MT, the resulting ZZ individuals will be genotypic and phenotypic males and WW individuals would have been either intersex individuals or appeared to be males based on external morphology. However, masculinization is rarely complete. It has been our experience in our laboratory and in our cooperative research with the Alaska Department of Fish and Game's Fort Richardson hatchery, masculinized female rainbow trout do not successfully form male reproductive ducts and sperm can only be obtained from them by removing the testes. Although masculinized female rainbow trout produce sperm, the

morphology of their gonads appears intermediate between testis and ovary. The degree to which WW individuals became masculinized would have depended on the strength and duration of the MT exposure. During segment 3, we evaluated several MT exposure regimes. In segment 4, we evaluated sex inversion using a new class of non-steroidal compounds called aromatase inhibitors that also indicate promise for masculinizing female fishes. A non-steroidal substitute for MT would be desirable due to drug enforcement considerations associated with anabolic steroids. Masculinized WW gynogens could have then been crossed with normal females. This would have resulted in WW and WZ progeny and both genotypes might have been female if a WZ/ZZ system were operational.

METHODS

1999 (Segment 1)

Broodstock acquisition, spawning procedures, and gynogenesis techniques were carried out as described in Job 101.1 (Segment 1) (above). The fish were trained to accept a prepared diet using the methods recommended by the staff at Jake Wolf Memorial State Fish Hatchery. When over 82 mm in total length the gynogens would be fed a diet containing methyltestosterone. The fish were to be placed in ponds containing fathead minnows as forage fish. Fish were to be reared until they reached sexual maturity after which their sperm would have been used to produce all-female offspring. Had the WZ/ZZ system been operative, then we needed only select incompletely masculinized individuals (WW individuals) for brood stock. We expected a low percentage of eggs to survive, probably less than 5 percent.

The perceived advantage of using northern pike sperm was that northern pike spawn a few weeks before muskellunge. The adult males could be maintained in the hatchery until needed, or their sperm can be held in flasks under refrigeration for several weeks. Northern

pike sperm is known to be compatible with muskellunge eggs since hybrids (tiger muskellunge) are frequently produced in fish hatcheries. The fish that survived from crosses with irradiated (to destroy the DNA) northern pike sperm (presumptive gynogens) were tested for species specific allozymes (Casselman et al. 1986). Any fish containing northern pike DNA would be a hybrid, not a diploid gynogen and, therefore, would be discarded. Hybrids can be produced, if the irradiation process is incomplete and does not destroy the DNA in all the sperm cells.

2000 (Segment 2)

Broodstock acquisition, spawning procedures, and gynogenesis techniques were carried out as described in Job 101.1 (Segment 2) (above). The fish were trained to accept a prepared diet, silver cup walleye grower 9206 (Nelson and Sons Inc., Murray, Utah), using the methods recommended by the staff at Jake Wolf Memorial State Fish Hatchery. When over 82 mm in total length 450 gynogenic muskellunge were separated into six rearing tanks (75 per tank), three of which were on a separate water supply from the other three. The fish in one group of three tanks were fed the prepared diet that had been fortified with 15 mg of 17α-methyl-testosterone (MT) per kg of the diet for 60 days. The MT was applied to the feed ahead of time by dissolving it in ethyl alcohol and spraying on the surface of the feed. The alcohol was then allowed to evaporate away, and the feed was packed in airtight containers for storage in a refrigerator. The fish in the other three tanks (controls) received the same diet that had not been fortified with MT.

After the 60 days of MT exposure the fish were transitioned on to live fathead minnows as forage fish and continued to be reared in a water-reuse system. When they had reached, approximately eight inches, a sample of the fish were sacrificed to determine histologically the

effect of the MT treatment on their gonads. Histological sections of the gonads were cut at 4μm and stained with Mayer's hematoxylin and counter-stained with eosin. Slides were examined in a blind fashion to control for observer bias. These fish were to be reared until they reached sexual maturity after which their sperm was to be used to produce all-female offspring. Had the WZ/ZZ system be operative, then we needed only select incompletely masculinized (intersex) individuals (WW individuals) for brood stock.

2001 (Segment 3)

Broodstock acquisition, spawning procedures, and gynogenesis techniques were carried out as described in Job 101.1 (Segment 3)(above). A portion of the eggs obtained from Jake Wolf State Fish Hatchery were used in an investigation designed to evaluate different pressure shock treatments to optimize production of gynogenic muskellunge. Five replicates of 12 different combinations of shock initiation times and shock durations were tested and each replicate had at least 200 eggs. Ten combinations tested pressure shocks and initiation times for meiotic gynogens, and two combinations tested initiation times for mitotic gynogenesis. For mitotic gynognesis, both $1.2\tau_o$ and $1.4\tau_o$ post fertilization shock times were tested, where τ_o is equal to the time it takes muskellunge eggs to undergo the first cleavage after fertilization (Lin and Dabrowski 1998).

Once hatched, the fish were trained to accept a prepared feed, (Silver Cup Walleye Grower 9206 Nelson and Sons Inc., Murray, Utah), using the methods recommended by the staff at Jake Wolf Memorial State Fish Hatchery. Once the gynogens were larger than 82 mm total length (TL), 228 of these fish were stocked into six rearing tanks (38 per tank); three of the rearing tanks were on a separate water supply from the other three. Fish in the first

designated three tanks were fed the prepared diet that had been fortified with 30 mg of 17α-methyl-testosterone (MT) per kg of feed for 60 days.

The MT was dissolved in ethanol and applied by spraying it on the surface of the feed. The alcohol was then allowed to evaporate and the feed was packed in airtight containers for refrigerated storage. The diet given to the fish in the remaining three tanks was the same except that it contained 50 mg of MT per kg of feed. A control batch of 114 fish distributed in an additional three tanks, were fed the same diet without MT. Control fish were also reared with a separate water supply from the other two treatments. A pilot study designed to test the lethal concentration of a non-steroidal aromatase inhibitor on 10 gynogenetic muskellunge was also conducted in a separate water recirculating system. After 60 days of MT exposure, fish were transitioned to live fathead minnows *Pimephales promelas* as forage fish and were reared in a different culture system.

2002 (Segment 4)

Broodstock acquisition, spawning procedures, and gynogenesis techniques were carried out as described in Job 101.1 (Segment 3)(above). A portion of the eggs obtained from Jake Wolf State Fish Hatchery were used in an investigation designed to evaluate different pressure shock treatments to optimize production of gynogenic muskellunge. Five replicates of 12 different combinations of shock initiation times and shock durations were tested and each replicate had at least 200 eggs. Ten combinations tested pressure shocks and initiation times for meiotic gynogens, and two combinations tested initiation times for mitotic gynogenesis.

The pilot study designed to test the ability of a non-steroidal aromatase inhibitor, Chrysin, to induce sex reversal in gynogenetic muskellunge was also conducted in a separate water recirculating system. Twenty-five (approximately 94 mm total length) fingerling

gynogenetic muskellunge were exposed to 1000 mg chrysin per kg of feed at a rate of 5% of body weight per day. After 60 days of Chrysin exposure, fish were transitioned to live fathead minnows *Pimephales promelas* as forage fish and were reared in a different culture system. Control, untreated, gynogenetic muskellunge were reared concurrently with a separate water supply that was maintained at the same temperature. After reaching a size appropriate for sex determination, the fish were sacrificed for histological examination of their gonads. Methods for histological preparation were as described previously.

RESULTS AND DISCUSSION

1999 (Segment 1)

Insufficient production of gynogenetic fish prevented production of any masculinized females. However, an accessory study was initiated to determine alternative egg activation techniques for the production of gynogens.

2000 (Segment 2)

A subsample of 24 gynogenic muskellunge that were fed MT showed a sex ratio of 17% females, 29% males, 17% immature females, and 37% inter-sex fish (Table 9). This compares to the 89% females to 11% males sex ratio of non-MT treated gynogenic muskellunge (n=28). The low percentage of males is a fortunate circumstance if the sex control mechanism of the muskellunge were WZ (See Job 101.1 Segment 2). Intersex individuals are desirable for back crossing to produce all female progeny, as they are potential WW genotypes if the WZ/ZZ system is operational (Job 101.3). The rest of these individuals were being raised in our facility until they reached maturity. At that time we hoped to attempt to spawn them with normal muskellunge to determine whether we can produce all-female progeny via this method.

2001 (Segment 3)

Fifteen-minute pressure shocks executed 5 to 10 minutes post fertilization yielded the greatest larval hatching rates (Tables 10 and 11). We did not find significant differences in survival among pressure shocking regimes for meiotic gynogenesis, although this was somewhat expected given muskellunge gynogenesis survival rate is only around 3% (Pandian and Koteeswaran 1998). Mitotic gynogenesis yielded only one larval fish at $1.2\tau_o$ (Table 12).

Eight gynogenic muskellunge were released in our reservoir at Touch of Nature and nine gynogenic muskellunge were released in a borrow pit at Logan Hollow Fish Farm for grow out. When mature (first possible spawning anticipated for 2004), we hope to attempt to spawn the gynogenetic fish with normal muskellunge to determine whether they produce all-female progeny. If the sex determination system is not a simple monofactorial XX/XY or WZ/ZZ, then complex cross-breeding studies will be required to identify individuals that yield a greater proportion of females to males.

Unfortunately, treatment groups from segment 3 received a lethal exposure of free chlorine and chloramines from city water during a routine system flushing due to a malfunction in the de-chlorination system. All individuals were taken from each treatment group post mortem for histological analysis of reproductive tissue to determine the effects of the two dosages of MT and Chrysin on the gonads. Histological sections of the gonads were cut at 4μm and subsequently stained with Mayer's hematoxylin and counter-stained with eosin. The individuals, however, had not yet matured enough for accurate sex identification. It was found muskellunge could tolerate up to 1000 mg Chrysin, a non-steroidal aromatase inhibitor, per kg of feed without any mortality. Higher dosages were not tested, because Chrysin at this concentration became saturated in the maximum volume of ethanol appropriate for treating a

given lot of feed. Increasing the volume of alcohol used to treat a given lot of feed causes excessive leaching of fats from the prepared diet.

2002 (Segment 4)

The optimum shock treatment identified during trials during spring 2002 (Segment 4) was once again a pressure treatment of 7000 psi applied 10 minutes after fertilization for a duration of 15 minutes (Tables 13 and 14). Once again, we did not find significant differences in survival among pressure shocking regimes for meiotic gynogenesis (see discussion for segment 3). Overall the years of this study (data from segments 2, 3, and 4) this pressure shock (7000 psi, 10 minute PFT, and 15 minute duration) had the highest percentage yield of gynogenetic progeny at hatch (Figure 2). Still, this relationship was not statistically significant due to high within replicate variability (the error term) and low overall survival rates.

The chrysin treatment failed to produce complete sex reversal of gynogenetic muskellunge (Table 15). However, it did undoubtedly show sex altering activity as evidenced by the number of intersex individuals produced. It may be that application of chrysin in the feed earlier in development of the fingerlings may have had the desired effect. Other non-steroidal aromatase inhibitors with higher activity (e.g., Fadrozole™ CGS16949A), may also be useful for masculinization (Kwon et al. 2002). However, in the context of this study, given the results of Job 101.1 Segment 4, the use of sex reversal for masculinization does not appear to be a method necessary for the production of all-female stocks of muskellunge at this time.

Job 101.3: Production of intersexed muskellunge broodfish

Objective: To produce intersexed broodfish, rear them to sexual maturity and use sperm from their testes to produce all female progeny.

Another method of producing all-female muskellunge, had the XX/XY system held (Job 101.1), was to feed juvenile muskellunge *Esox masquinongy* a prepared diet that contained methyltestosterone (MT) to masculinize genetic females, producing intersexed individuals, i.e., individuals that have both testes and ovaries. Individuals with only testes are genotypic males. Sperm in the intersex fish would carry only the X female chromosome. With this approach, the intersex fish are sacrificed at sexual maturation because of the incomplete formation of the sperm duct and, in turn, the improbability of traditional manual milting. The testes are macerated, an extender is added, and the mixture is used to fertilize the eggs producing all-female offspring. This procedure has not been successfully conducted with muskellunge though it has been conducted successfully with walleye *Stizostedion vitreum* (Malison et al. 1998).

In walleye, Malison et al. (1998) produced intersex specimens only when 15 mg of MT per kg of food was fed to 50 mm fingerlings. They found this was the size where oogenesis commenced. Fish treated at 75 mm and 100 mm total length (TL) did not develop the intersexed condition. Subsequently, Malison (unpublished data) has found manipulating dosage of MT given to fish at an appropriate size for sex-reversal is more appropriate for optimizing production of inter-sex fish when the size at oogenesis is known. The size range where oogenesis is reported to be beginning in muskellunge is between 82 mm and 138 mm (Lin et al. 1997). Dabrowski (unpublished) has identified this as the appropriate size range for inducing sex-reversal in muskellunge.

If the WZ/ZZ system was in operation, we anticipated that ZZ individuals would have appeared as males after exposure to methyltestosterone in all treatment regimes. We also

anticipated that WW individuals would show intersex characteristics after exposure to one or more of the methyltestosterone treatments regimes. If so, this would have enabled us to select intersex, WW individuals for broodfish. When crossed with normal WZ females, WW and WZ progeny would be produced, and we anticipated that both genotypes would be female.

METHODS

1999 (Segment1)

Adult muskellunge broodfish were stripped of gametes using standard culture procedures by personnel at Jake Wolf Memorial State Fish Hatchery (Westers and Stickney 1993). The offspring were trained to accept a standard prepared diet. When they reached a mean size of 88 mm, four tanks with 60 individuals per tank were fed diets containing 11.25, 7.5, 3.75, and 0 mg of methyltestosterone (MT) per kilogram of food (mg MT/Kg) for 60 days. The three dosages of MT treated feed correspond to 75%, 50%, and 25% of the typical sex reversing dosage of 15 mg MT/Kg of feed. A group of control fish, fed untreated feed 0 mg MT/Kg, were maintained simultaneously for sex ratio comparisons.

Initially, treatment lots of fish were kept separately in indoor rearing tanks. After completion of MT treatment the fish were weaned off of the prepared diet and on to fathead minnows. Subsequently, they were moved into ponds containing fathead minnows as forage at our 90-pond unit research facility. Representatives of the experimental treatments were to be reared to sexual maturity and their sperm used to produce all-female offspring. A sample of 10 individuals was taken prior to treatment, and from each treatment at the end of the MT treatment period for histological analysis of gonadal material.

2000 (Segment 2)

Adult muskellunge broodfish were stripped of gametes using standard culture procedures by personnel at Jake Wolf State Fish Hatchery (Westers and Stickney 1993). The offspring were trained to accept a standard prepared diet. When they reached a mean size of 4 in (100 mm), three replicate tanks each of three MT treatments and a control were stocked with 50 individuals. The MT treatments were applied to a prepared diet at rates of 11.25, 7.5, and 3.75 of MT/kg of feed. The fish were fed the MT fortified diet for 60 days. The control fish were fed the same diet that was not fortified with MT. The three dosages of MT treated feed correspond to 75%, 50%, and 25% of a typical sex reversing dosage of 15 mg MT/Kg of feed.

Initially, treatment lots of fish were kept separately in indoor rearing tanks. After completion of MT treatment the fish were weaned off of the prepared diet and on to fathead minnows. After the optimum treatment to induce the inter-sex state is determined they were to be moved into ponds containing fathead minnows as forage at our 90-pond unit research facility. Representatives of the experimental treatments were to be reared to sexual maturity and their sperm used to produce all-female offspring. A sample of 10 individuals per tank was taken when the fish had reached a size of 7 to 8 in to determine histologically the effect of the MT treatment on their gonads. Histological sections of the gonads were cut at 4μ and stained with Mayer's hematoxylin and counter-stained with eosin. Slides were examined in a blind fashion to control for observer bias.

A pilot study looking at pit tag retention was conducted using fish obtained from Jake Wolf State Fish Hatchery. We wanted to know whether 6-8 inch muskellunge could be marked successfully with pit tags so that the experimental groups could be combined in larger ponds for grow-out. We also wanted to know where the pit tag should be implanted on a

fingerlings body. Initially nine muskellunge fingerlings were implanted with pit tags, three ventrally in the body cavity in between the pectoral fins, three ventrally in the body cavity in between the pelvic fins, and three in the muscle below the dorsal fin. The best of these three treatments was used in a month long study with 15 fish to see if excessive mortalities occurred, or if the pit tags were not retained.

2001 (Segment 3)

Initially, treatment lots of fish from Job 101.3 segment 2 were kept separately in indoor rearing tanks. After completion of MT treatment during segment 3, the fish were weaned off the prepared diet and on to fathead minnows. Fourteen representatives of the MT experimental treatments were being reared on white suckers until they reached sexual maturity. These fish will not be sexually mature until they are at least three years old (the fiscal year after segment 4 was to be completed), at which time their sperm was be used in attempts to produce all female offspring.

2002 (Segment 4)

No work was scheduled for this segment.

RESULTS AND DISCUSSION

1999 (Segment 1)

A total of 16 fish from the 11.25 mg MT/Kg treatment were split between two ponds, 27 from the 7.5 mg MT/Kg treatment were split between two ponds, and 27 from the 3.75 mg MT/Kg treatment were split between two ponds for a total of six stocked ponds. Each pond was pre-stocked with fathead minnows as forage. At the end of the MT treatment period the mean total length of each group ranged from 140.4 to 149.5 (Table 15). There were no

significant differences in length or weight (ANOVA; alpha=0.05) between the treatment groups at the end of the 60 day treatment period.

2000 (Segment 2)

Histological examination of samples taken from muskellunge fingerlings produced during 1999 (Segment 1) revealed that the fish were large enough to distinguish males from females but not to distinguish whether the gonads of any of the females had been effected by the MT treatment sufficiently to be classified as intersex. This is why the muskellunge fingerlings used for the tests during year 2000 (Segment 2) were allowed to grow to a larger size than the year 1999 (Segment 1) fingerings prior to sampling. Examination of the year 2000 (Segment 2) intersex study individuals showed an interesting response to the addition of MT to their diet (Figure 1). From these results it appears that as MT dosage increases the proportion of females increases until a certain level of MT is reached at which point mostly males and intersex individuals are produced. It is odd to see such a threshold effect rather that the expected dose response relationship, wherein as MT is increased the proportion of males and intersex individuals increases. Rinchard et al (1999) reported paradoxical feminization of muskellunge that were fed a diet that contained 15 mg MT/ kg of feed. So, the effect of MT on the gonads of muskellunge is not certain. However, 11.25 mg MT/ kg feed did result in a mean of 16.7% intersex individuals in our study. Rinchard et al. (1999) also noted a change in sex ratio over time in the population of fish that was being studied. Therefore, we hoped to resample our fish when they reached a larger size to see if the proportions of males, females and intersex fish remain consistent. Rinchard et al. (1999) suggested that alternate treatments, such as 17α-methyldihydrotestosterone, be applied to affect sex reversal.

The pilot study of pit tag retention revealed that implantation of a pit tag under the dorsal fin and on the ventral side between the pectoral fish is unsatisfactory, due to lack of tag retention and excessive mortality, respectively (Table 16). Implantation of the pit tag ventrally between the pelvic fins resulted in 66% retention and 66% mortality. This treatment was further tested in a month-long trial with 15 tagged individuals. The mortality rate at the end of the month-long trial was only 7% (n=1) and all 15 had retained their implanted pit tags. At the start of the trial the mean size of the fingerlings was 6.4 in and at the end the mean size was 7.6 in a change of more than 1 inch. So, implantation of the pit tag did not prevent muskellunge fingerlings from growing. The optimum site for implantation of pit tags in fingerling muskellunge appears to be in between the pelvic fins.

2001-2002 (Segments 3and 4)

This procedure was applicable if the XY sex determination system operated in the muskellunge. However, evidence obtained by us and others during the progress of these research lines indicated that the XY genetic sex-determination system is not evident in mukellunge. Consequently, we shifted effort from this job (101.3) to job 101.1 during Segments 3 and 4.

Job 101.4: Preparation of reports

Objectives: To analyze the results of Jobs 101.1, 101.2, and 101.3 annual and completion reports as required by the U.S. Fish and Wildlife Service and IDNR.

This project completion report is submitted to satisfy the requirements of this job.

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Table 1. Production and viability of putative gynogenic muskellunge at Jake Wolf Memorial State Fish hatchery during spring of 1999 (Segment 1).

Date	Fish Number	Volume of eggs mL	Number of eggs[a]	% Viablilty at eyed stage	Number of swimup fry	% return
3/4/99	1	120	5892	44	10	0.17
3/5/99	2	100	4910	51.8	264	2.11
	3	75	3682.5	53.7	-[b]	-[b]
	4	100	4910	0.7	0	0
	5	100	4910	1.7	0	0
	6	80	3928	83	-[b]	-[b]
3/6/99	7	150	7365	64.4	66	0.33
	8	140	6874	61.6	-[c]	-[c]
	9	115	5646.5	-[c]	-[c]	-[c]
3/8/99	10	100	4910	2.1	0	0
3/9/99	11	125	6137.5	n/a	25	0.07
	12	165	8101.5	n/a	-[d]	-[d]
	13	100	4910	n/a	-[d]	-[d]
	14	240	11784	n/a	-[d]	-[d]
	15	125	6137.5	n/a	-[d]	-[d]
Total		1835	90098.5		365	0.41

[a/] Based on a mean size of 49.1 eggs per mL.

[b/] Groups 2, 3 and 6 mixed to conserve tank space.

[c/] Groups 7, 8 and 9 mixed to conserve tank space.

[d/] Groups 11 through 14 mixed to conserve tank space.

Table 2. Number of broodfish captured each day of sampling from Kinkaid Lake, number of ripe females, and production of putative gynogenic muskellunge during spring of 1999 (Segment 1).

Date	Male	Female	Immature	Total	Ripe Females	Volume of eggs mL	Number of eggs[a]	Number of swimup fry	% return
3/23/99	12	1	1	14	0	-	-	-	-
3/24/99	6	5	0	11	0	-	-	-	-
3/25/99	11	8	0	19	0	-	-	-	-
3/26/99	16	3	0	19	0	-	-	-	-
3/29/99	5	2	0	7	0	-	-	-	-
3/30/99	15	5	0	20	0	-	-	-	-
3/31/99	8	2	0	10	0	-	-	-	-
4/1/99	5	4	0	9	1	100	4910	43	0.88
4/2/99	4	5	0	9	0	-	-	-	-
4/5/99	16	8	0	24	1	1000	49100	0	0
4/6/99	9	9	2	20	0	-	-	-	-
Total	107	52	3	162	2	1100	54010	43	0.08

[a/] Based on a mean size of 49.1 eggs per mL.

Table 3. Production and viability of gynogenic muskellunge at Jake Wolf Memorial State Fish hatchery during spring of 2000 (Segment 2). Ultraviolet-irradiated walleye spermatozoa were used to activate the muskellunge eggs.

Date	Treatment[a]	UV[b] (J/m^2)	PFT[c] (min)	Shock Duration (min)	Control Hatch Rate	Egg Volume (ml)	Number of Eggs	Number of Fry	% Actual Survival	% Survival Relative to Control	Number of 3 inch fingerlings
3/6/00	Heat	648	20	10	0.55	360	18000	9	0.05	0.09	29[e]
	Pressure	648	15	3	0.55	110	5500	133	2.42	4.40	--
	Pressure	648	20	3	0.55	110	5500	28	0.51	0.93	--
3/8/00	Heat	M[d]	20	10	0	50	2500	0	0.00	0.00	--
	Pressure	M[d]	15	3	0.012	60	3000	16	0.53	44.44	--
	Pressure	M[d]	20	3	0.012	75	3750	33	0.88	73.33	--
	Heat	M[d]	30	10	0.012	75	3750	1	0.03	2.22	--
3/9/00	Heat	648	20	10	0.132	50	2500	0	0.00	0.00	--
	Pressure	648	27	3	0.132	50	2500	0	0.00	0.00	--
	Pressure	648	22	3	0.132	50	2500	3	0.12	0.91	--
	Pressure	648	17	3	0.132	45	2250	1	0.04	0.34	--
	Pressure	648	20	3	0	225	11250	0	0.00	0.00	--
3/10/00	Heat	M[d]	20	3	0.25	190	9500	0	0.00	0.00	--
	Pressure	648	20	3	0.67	150	7500	3	0.04	0.06	--
	Pressure	2160	20	3	0.67	160	8000	0	0.00	0.00	--
	Heat	648	20	10	0.46	90	4500	0	0.00	0.00	--
	Heat	2160	20	10	0.46	90	4500	0	0.00	0.00	--
	Pressure	648	15	3	0.46	90	4500	0	0.00	0.00	--
	Pressure	2160	15	3	0.46	90	4500	4	0.09	0.19	--
	Heat	M[d]	20	10	0.58	300	15000	1	0.01	0.01	--
	Pressure	M[d]	20	3	--	100	5000	34	0.68	--	--
3/11/00	Pressure	M[d]	20	3	0.38	300	15000	554	3.69	9.72	76
	Heat	M[d]	20	10	0.56	220	11000	8	0.07	0.13	--
TOTAL						3040	152000	828			105
MEAN					0.33				0.40	6.22	
STD					0.24				0.90	17.76	

(Table 3. Cont.)

[a/] Hydrostatic pressure shocks were all conducted at 7000 psi; heat shocks were all conducted at 86° F (30° C).

[b/] Amount of Ultraviolet irradiation sperm received.

[c/] Time of shock initiation after fertilization.

[d/] 50:50 mixture of sperm irradiated at 2160 J/m^2 and 648 J/m^2 .

[e/] Includes all fry except those produced from the pressure shock on 3/11/00.

Table 4. Number of broodfish captured each day of sampling from Kinkaid Lake, number of ripe females, number of females spawned, production of gynogenic muskellunge fry, and 3 inch gynogenic muskellunge fingerlings during spring of 2000 (Segment 2). Ultraviolet-irradiated (648 J/m^2) yellow perch sperm was used to activate the muskellunge eggs.

Date	Male	Female	Immature	Total	Number of Ripe Females	Number Spawned[a]	Volume of eggs mL	Number of eggs	Number of fry	% return	Number of 3 inch fingerlings
3/7/00	9	0	1	10	0	0	-	-	-	-	-
3/8/00	15	2	1	18	0	0	-	-	-	-	-
3/9/00	14	5	2	21	0	0	-	-	-	-	-
3/13/00	1	1	0	2	0	0	-	-	-	-	-
3/14/00	0	2	0	2	0	0	-	-	-	-	-
3/15/00	5	2	0	7	0	0	-	-	-	-	-
3/16/00	4	2	0	6	0	0	-	-	-	-	-
3/17/00	2	3	0	5	0	1	200	10000	0	0	0
3/18/00	1	1	1	3	0	0	-	-	-	-	-
3/19/00	3	1	0	4	0	0	-	-	-	-	-
3/20/00	4	6	0	10	1	3	1950	97500	294	0.30	56
3/21/00	11	3	1	15	0	1	800	40000	300	0.75	48
3/22/00	9	6	1	16	2	2	1360	68000	915	1.35	347
Total	78	34	7	119	3	7	4310	215500	1509	0.70	451

[a/] Includes individuals captured on earlier days that were injected with carp pituitary to stimulate ovulation

[b/] Based on a egg size of 50 eggs per mL.

Table 5. Gynogenesis pressure shocking regimes and distribution of gender within six broods of gynogenetic muskellunge *Esox masquinongy* produced during Spring 2002 (segment 4) from six females captured from Spring Lake, Manito, Illinois. Female identification number (Female), time elapsed in minutes from the activation of ova to the commencement of the pressure shock (PFT), duration of the 7000 PSI pressure shock in minutes (DS), total eggs undergoing gynogenesis from each female (Eggs), number of gynogenetic fish hatched (Hatch), number of males, number of females, and percent females in each brood.

Female	PFT	DS	N_t	N_M	N_F	%Female
63E77	10	15	16	0	16	100
47F79	5	15	7	3	4	57
5C3A	[a]	[a]	15	0	15	100
684A	10	15	6	2	4	67
70822	10	15	5	0	5	100
11809	5	15	9	0	9	100
Total				5	53	93

[a/] Eggs from female 5C3A were divided among three pressure shocking regimes: PFT 5, Shock 15; PFT 10, Shock 15; and PFT 20, Shock 3. These eggs were compiled to obtain the sex ratio for the brood.

Table 6. AFLP primer combinations and product results for muskellunge (n=11). Primers are listed as E-www or M-xxx, where E- and M- designate the *Eco*RI or *Mse*I restriction endonucleases and www or xxx are the selective base extensions on the 3' end of each primer, respectively. The number of polymorphic fragments and total fragments generated from the AFLP are listed along with the proportion of polymorphic fragments for each primer combination.

*Eco*RI Primer	*Mse*I Primer	Polymorphic Fragments	Total Fragments	Proportion Polymorphic
E-ACA	M-CTA	1	40	0.0250
E-ACA	M-CTC	1	44	0.0227
E-ACA	M-CTG	6	88	0.0682
E-ACA	M-CTT	4	44	0.0909
E-ACA	M-CAA	*	*	*
E-ACA	M-CAG	7	70	0.1000
E-ACA	M-CAT	1	61	0.0164
E-ACA	M-CAC	*	*	*
E-ACG	M-CTA	11	50	0.2200
E-ACG	M-CTC	*	*	*
E-ACG	M-CTG	10	71	0.1408
E-ACG	M-CTT	7	48	0.1458
E-ACG	M-CAA	0	31	0
E-ACG	M-CAG	5	44	0.1136
E-ACG	M-CAT	6	41	0.1463
E-ACG	M-CAC	8	39	0.2051
E-ACT	M-CTA	1	67	0.1049
E-ACT	M-CTC	2	43	0.0465
E-ACT	M-CTG	4	73	0.0548
E-ACT	M-CTT	5	71	0.0704
E-ACT	M-CAA	3	79	0.0380
E-ACT	M-CAG	3	52	0.0577
E-ACT	M-CAT	3	62	0.0484
E-ACT	M-CAC	1	45	0.0222
E-AAC	M-CTA	4	72	0.0556
E-AAC	M-CTC	7	48	0.1458
E-AAC	M-CTG	*	*	*
E-AAC	M-CTT	1	58	0.0172
E-AAC	M-CAA	3	82	0.0366
E-AAC	M-CAG	1	81	0.0123
E-AAC	M-CAT	0	41	0
E-AAC	M-CAC	4	68	0.0588

* Indicates locus could not be reliably scored

Table 6. (continued)

*Eco*RI Primer	*Mse*I Primer	Polymorphic Fragments	Total Fragments	Proportion Polymorphic
E-AGC	M-CTA	6	47	0.1277
E-AGC	M-CTC	6	43	0.1395
E-AGC	M-CTG	13	40	0.3250
E-AGC	M-CTT	5	64	0.0781
E-AGC	M-CAA	6	57	0.1053
E-AGC	M-CAG	3	59	0.0508
E-AGC	M-CAT	0	61	0
E-AGC	M-CAC	4	54	0.0741
E-ACC	M-CTA	6	49	0.1224
E-ACC	M-CTC	7	47	0.1489
E-ACC	M-CTG	5	37	0.1351
E-ACC	M-CTT	3	43	0.0698
E-ACC	M-CAA	4	56	0.0714
E-ACC	M-CAG	3	42	0.0714
E-ACC	M-CAT	0	33	0
E-ACC	M-CAC	6	43	0.1400
E-AAG	M-CTA	*	*	*
E-AAG	M-CTC	*	*	*
E-AAG	M-CTG	*	*	*
E-AAG	M-CTT	*	*	*
E-AAG	M-CAA	2	64	0.0312
E-AAG	M-CAG	1	60	0.0167
E-AAG	M-CAT	0	62	0
E-AAG	M-CAC	3	57	0.0526
E-AGG	M-CTA	3	45	0.0667
E-AGG	M-CTC	6	51	0.1176
E-AGG	M-CTG	6	63	0.0952
E-AGG	M-CTT	2	53	0.0377
E-AGG	M-CAA	*	*	*
E-AGG	M-CAG	*	*	*
E-AGG	M-CAT	*	*	*
E-AGG	M-CAC	*	*	*

* Indicates locus could not be reliably scored.

Table 7. Polymorphic microsatellite loci in female muskellunge used to generate gynogenetic progeny. Loci, core sequences, sample size (N), optimal annealing temperature (T_a), number of alleles per locus N_A, product size ranges given in base pairs, observed heterozygosity (H_o), and 5'-3' PCR priming sequences. For *Elu*51, the smallest muskellunge product was scored as 121.

Locus	Core Sequence	N	T_a	N_A	Product Size Range	H_o	Priming Sequence (5'-3')
*Ema*3	$(CA)_9N_4(CA)_3N_2(CA)_3N_{29}$-$(TC)_4N_3(CA)_{15}$	8	60°C	5	197-209	0.750	F: CAGTCCATTCAGGGGGTATG R: CACCTGTGTGAGTGTGTGACC
*Ema*13	$(TG)_{17}$	8	60°C	6	104-122	0.250	F: CACGCTCTAGTGAACACGTCTCC R: TGTTGTCCCTCAACTTCCATTAACC
*Ema*15	$(TG)_{13}C(TG)_{12}$	8	64°C	3	149-153	0.750	F: GAGCCTCTGAAGGAATCAGGATG R: TAGTGACTCCAACTCCTCTCTCTCG
*Ema*30	$(TG)_{14}$	8	64°C	3	125-129	0.625	F: TATGCCAAATGGCTCCTCTAATG R: AAGTGCCCGCAGGAACGTCAAC
*Ema*31	$(TC)_7(AC)_{12}$	8	64°C	5	155-179	1.000	F: CAGAACACGCTTTACAAAGCAGG R: AGTCTCCAAATCCCACAGTGGAC
*Ema*32	$(TG)_{22}(TCTG)_6(TG)_{11}$-$CTTT(GT)_6$	8	60°C	7	206-260	0.875	F: TACCCCATTGGTCTGGTGG R: AAGTTCTAACAAGTAGCCTCTCCC
*Elu*51	$(AC)_{16}$	8	60°C	2	121-125	0.250	F: GTGGGCATTCAGCCGATATAGC R: CTGTCTCATTACTGCCTGGCTC

Core sequences and primer sequences for *Elu*51 were taken from Miller and Kapuscinski (1996) and *Ema*3, *Ema*13, *Ema*15, *Ema*30, *Ema*31, and *Ema*32 were taken from Reading et al. (2003).

Table 8. Number of non-recombinant: recombinant gynogens at each microsatellite locus tested. Loci (along top), female identification numbers (Female), total number of gynogenetic broods tested for each locus (N_B), average observed heterozygosities (H_O), standard deviation of H_O (SD), Roff and Bentzen (1989) χ^2 value for frequency of recombinants between broods, and p of χ^2 test. Overall observed heterozygosity of all gynogens across all loci was 0.576 (SD=0.237). None of the χ^2 tests were significant after a sequential Bonferroni adjustment of alpha.

Female Number	*Ema*15	*Ema*30	*Ema*31	*Elu*51	*Ema*13	*Ema*3	*Ema*32
63E77	3:17	a	15:5	2:18	a	20:0	7:13
684A	3:17	6:14	10:10	a	3:17	19:0	a
65720	6:9	5:10	11:4	a	6:9	14:1	7:8
C1827	2:17	1:18	19:0	4:14	a	a	5:14
5B0F	4:16	a	15:4	a	a	17:2	7:13
47F79	6:11	a	14:3	a	a	a	10:7
70822	a	3:17	15:5	a	a	20:0	a
5C3A	a	3:10	10:3	a	a	11:1	3:10
N_B	6	5	8	2	2	6	6
H_O	0.775	0.787	0.236	0.839	0.725	0.043	0.625
SD	0.121	0.114	0.137	0.086	0.177	0.048	0.133
p	0.213	0.220	0.059	0.190	0.048	0.281	0.275

[a/] Denotes female was homozygous for that locus.

Table 9. Sex ratio of gynogenic muskellunge fed a prepared diet containing 15mg 17α-methyltestosterone (MT) / kg of feed, and gynogenic muskellunge fed the same diet that was not treated with MT.

	Untreated Gynogens		MT treated Gynogens	
Gender	n	Percent	n	percent
Female	25	89	8	34
Male	3	11	7	29
Intersex	0	0	9	37

Table 10. Production and viability of gynogenic muskellunge from Jake Wolf Memorial State Fish Hatchery during spring of 2001 (Segment 3). Ultraviolet irradiated yellow perch spermatozoa were used to activate the muskellunge eggs.

Female Number	Pressure Treatment	UV (J/m2)	Shock Duration (min)	PFT (min)	Number of Eggs	Number of Eyed Eggs	Number Of Fry Produced
4B12	7000psi	684	15	5	546	181	1
5C3A	7000psi	684	15	5	506	432	4
5041	7000psi	684	15	5	279	52	9
6633	7000psi	684	15	5	342	77	10
6427	7000psi	684	15	5	351	111	4
4B12	7000psi	684	15	10	476	103	3
5C3A	7000psi	684	15	10	622	543	4
5041	7000psi	684	15	10	283	57	19
6633	7000psi	684	15	10	286	82	10
6427	7000psi	684	15	10	347	76	1
4B12	7000psi	684	10	5	635	170	6
5C3A	7000psi	684	10	5	553	508	1
6633	7000psi	684	10	5	376	84	0
5041	7000psi	684	10	5	327	22	0
6427	7000psi	684	10	5	340	80	3
4B12	7000psi	684	10	10	512	112	0
5C3A	7000psi	684	10	10	503	395	11
6633	7000psi	684	10	10	203	38	5
6427	7000psi	684	10	10	272	72	0
4B12	7000psi	684	10	15	477	94	0
5C3A	7000psi	684	10	15	725	610	16
6633	7000psi	684	10	15	322	82	3
6427	7000psi	684	10	15	337	96	8
5041	7000psi	684	10	15	372	26	0
4B12	7000psi	684	5	10	516	169	0
5C3A	7000psi	684	5	10	621	415	2
5041	7000psi	684	5	10	318	36	6
6633	7000psi	684	5	10	339	95	3
6427	7000psi	684	5	10	350	61	3
4B12	7000psi	684	5	15	446	167	12
5C3A	7000psi	684	5	15	636	490	2
5041	7000psi	684	5	15	336	59	0
6633	7000psi	684	5	15	400	130	2
6427	7000psi	684	5	15	336	115	1

Table 10. (Continued)

Female Number	Pressure Treatment	UV (J/m2)[a]	Shock Duration (min)	PFT (min)[b]	Number of Eggs	Number of Eyed Eggs[c]	Number Of Fry Produced
4B12	7000psi	684	5	25	642	209	0
5C3A	7000psi	684	5	25	602	412	9
5041	7000psi	684	5	25	344	30	9
6633	7000psi	684	5	25	332	62	4
6427	7000psi	684	5	25	340	61	3
4B12	7000psi	684	3	15	540	223	2
5C3A	7000psi	684	3	15	614	550	4
5041	7000psi	684	3	15	318	48	16
6633	7000psi	684	3	15	362	72	0
6427	7000psi	684	3	15	322	57	1
4B12	7000psi	684	3	20	547	207	5
5C3A	7000psi	684	3	20	524	396	4
5041	7000psi	684	3	20	373	60	11
6633	7000psi	684	3	20	314	36	5
6427	7000psi	684	3	20	351	101	3

[a/] Amount of ultraviolet irradiation sperm received
[b/] Time of shock initiation after fertilization
[c/] Eyed eggs were counted at least 24 hours after fertilization.

Table 11. Summary of the viability of each treatment of meiotic gynogenesis of muskellunge from Jake Wolf Memorial State Fish Hatchery Spring 2001 (Segment 3).

Shock Duration (min)	PFT (min)[a]	% Survival to Eyed Egg ± SE	% Hatch from Eyed ± SE	% Actual Survival ± SE
15	5	38.26 ± 12.09	7.075 ± 3.406	1.652 ± 0.6024
15	10	35.93 ± 12.93	10.10 ± 6.167	2.354 ± 1.234
10	5	34.25 ± 14.81	1.495 ± 0.8769	0.4016 ± 0.2118
10	10	36.40 ± 14.13	3.986 ± 3.127	1.162 ± 0.6735
10	15	32.96 ± 13.31	2.923 ± 1.533	1.102 ± 0.5146
5	10	31.27 ± 9.661	5.045 ± 3.041	0.7902 ± 0.3209
5	15	39.75 ± 9.929	2.000 ± 1.321	0.7605 ± 0.4891
5	25	29.27 ± 10.51	8.711 ± 5.437	1.240 ± 0.4258
3	15	36.71 ± 14.01	7.342 ± 6.504	1.273 ± 0.9453
3	20	33.95 ± 11.40	7.724 ± 3.501	1.415 ± 0.4107

[a/] Time of shock initiation after fertilization

Table 12. Production and viability of mitotic gynogens from Jake Wolf Memorial Fish Hatchery during Spring 2001 (Segment 3). Ultraviolet-irradiated (648 J/m^2) yellow perch sperm was used to activate the muskellunge eggs. τ_o is equal to the time it takes muskellunge eggs to undergo the first cleavage after fertilization at a given temperature. All shocks were conducted at 7000 psi of hydrostatic pressure.

Female Number	PFT (τ_o)	Number of Eggs	Number of Eyed Eggs	Number of Fry	% Survival to Eyed Egg	% Survival to hatch
6427	1.2	313	72	0	23.0	0
6427	1.4	367	119	0	32.4	0
4B12	1.2	126	14	0	11.1	0
4B12	1.4	450	43	0	9.6	0
5C3A	1.2	131	36	1	27.5	0.008
5C3A	1.4	295	55	0	18.6	0
5041	1.2	376	18	0	4.8	0
5041	1.4	238	12	0	5.0	0
4B12	1.2	544	128	0	23.5	0
4B12	1.4	624	174	0	27.9	0
6633	1.2	367	69	0	18.8	0
6633	1.4	305	88	0	28.9	0
5C3A	1.2	634	410	0	64.7	0
5C3A	1.4	607	444	0	73.2	0

Table 13. Production and viability of gynogenetic muskellunge from Jake Wolf Memorial State fish Hatchery during Spring of 2002 (Segment 4). Ultraviolet irradiated (684 J/m^2) yellow perch spermatozoa were used to activate the muskellunge eggs. PFT = shock initiation time after fertilization. All shocks were done at 7000 psi.

Fish Number	Date Spawned	PFT (min)	Shock Duration (min)	Number of Eggs	Survival (Day 4)	% Survival (Day 4)	Number Hatched	% Hatched	Control % Hatched	Relative Percent Hatched
5C3A	3/15/2002	[a]	[a]	5867	1439	24.5	267	4.6	54.3	8.5
71D59	3/18/2002	10	15	4640	440	9.5	[b]	--	61.4	--
11809	3/18/2002	5	15	6850	1805	26.4	550	8.0	62.0	13.0
4F5B	3/18/2002	5	15	6190	1507	25.4	87	1.4	59.5	2.4
65C3A	3/18/2002	20	3	6930	1616	23.3	[b]	--	61.1	--
5B0F	3/18/2002	20	3	6311	1711	27.1	105	1.7	61.9	2.7
684A	3/18/2002	10	15	5727	1327	23.2	304	5.3	61.8	8.6
47F79	3/18/2002	5	15	4587	1307	28.5	560	12.2	63.3	19.3
63E77	3/18/2002	10	15	6390	3076	48.1	1159	18.1	58.1	31.2
7237	3/18/2002	20	3	5409	609	11.3	13	0.002	81.1	0.003
65720	3/19/2002	10	15	4806	1126	23.4	575	12.0	58.0	20.6
A2536	3/19/2002	5	15	4146	186	4.5	[b]	--	66.1	--
C1827	3/19/2002	20	3	6210	1414	22.8	333	5.3	61.3	8.8
55646	3/19/2002	5	15	4520	0	0.0	0	0.0	71.2	0.0
70822	3/19/2002	10	15	4525	685	15.1	214	4.7	63.1	7.5
Total				83108	18248	22.0	4167	5.0	62.9	8.0

[a] Three shock treatment groups combined due to rearing space available (PFT, Duration; 5,15; 10,15;and 20,3)

[b] Treatments combined due to rearing space available Total Yield at hatch of 158 sac fry

Table 14. Summary of the viability of each meiotic gynogenesis shock treatment (activated with ultraviolet irradiated (684 J/m^2) yellow perch spermatozoa and pressure shocked at 7000 psi) and controls (fertilized with muskellunge sperm and unshocked) of muskellunge from Jake Wolf Memorial State Fish Hatchery Spring 2002 (Segment 4).

Shock Duration (min)	PFT (min)[a]	N	% Survival to Eyed Egg (SD)	% Actual Survival to Hatch (SD)	% Survival of Controls to Hatch	% Relative Survival to Hatch (SD)
15	5	4	20.1 (13.4)	5.4 (5.7)	64.0 (5.1)	8.7 (9.1)
15	10	4	27.5 (14.3)	10.0 (6.3)	60.3 (2.6)	17.0 (11.2)
3	20	3	20.4 (8.2)	2.3 (2.7)	68.1 (11.3)	3.8(4.5)

[a/] Time of shock initiation after fertilization

Table 15. Sex ratio and total length (mm) (TL) of gynogenic muskellunge fed a prepared diet containing 1000 mg chrysin / kg of feed, and gynogenic muskellunge fed the same diet that was not treated with chrysin. Distribution of sexes between treatments are significantly different (Roff and Bentzen (1989) chi-square; $p \leq 0.0001$)

Treatment	N	TL (SD)[a]	Male	Female	Intersex
Untreated	9	124.7 (10.1)	1	8	0
Chrysin Treated	25	138.0 (10.1)	2	7	16

Table 15. Mean and standard deviation (SD) of total length (TL) and weight of muskellunge at the end of a 60 day period of being fed a prepared diet containing three levels of 17α-methyltestosterone (MT) (Segment 1). Treatments are expressed as mg of MT per Kg of prepared diet.

Treatment	Mean TL	SD	Weight (g)	SD
3.75	149.5	9.5	13.4	3.0
7.5	142.6	8.4	11.0	1.9
11.25	140.4	9.0	11.0	2.5

Table 16. Tag retention and number of mortalities as a result of pit tag implantation in three areas of the body of 6 to 8 in muskellunge fingerlings. Body areas for implantation were ventrally in between the pectoral fins (Pectoral), ventrally in between the pelvic fins (Pelvic), and below the dorsal fin (Dorsal). Duration of study was 30 days.

Tag Site	N	# mortalities	# survivors	# retained tags	# expelled tags
Pectoral	3	3	0	3	0
Pelvic	3	1	2	2	1
Dorsal	3	1	2	0	3

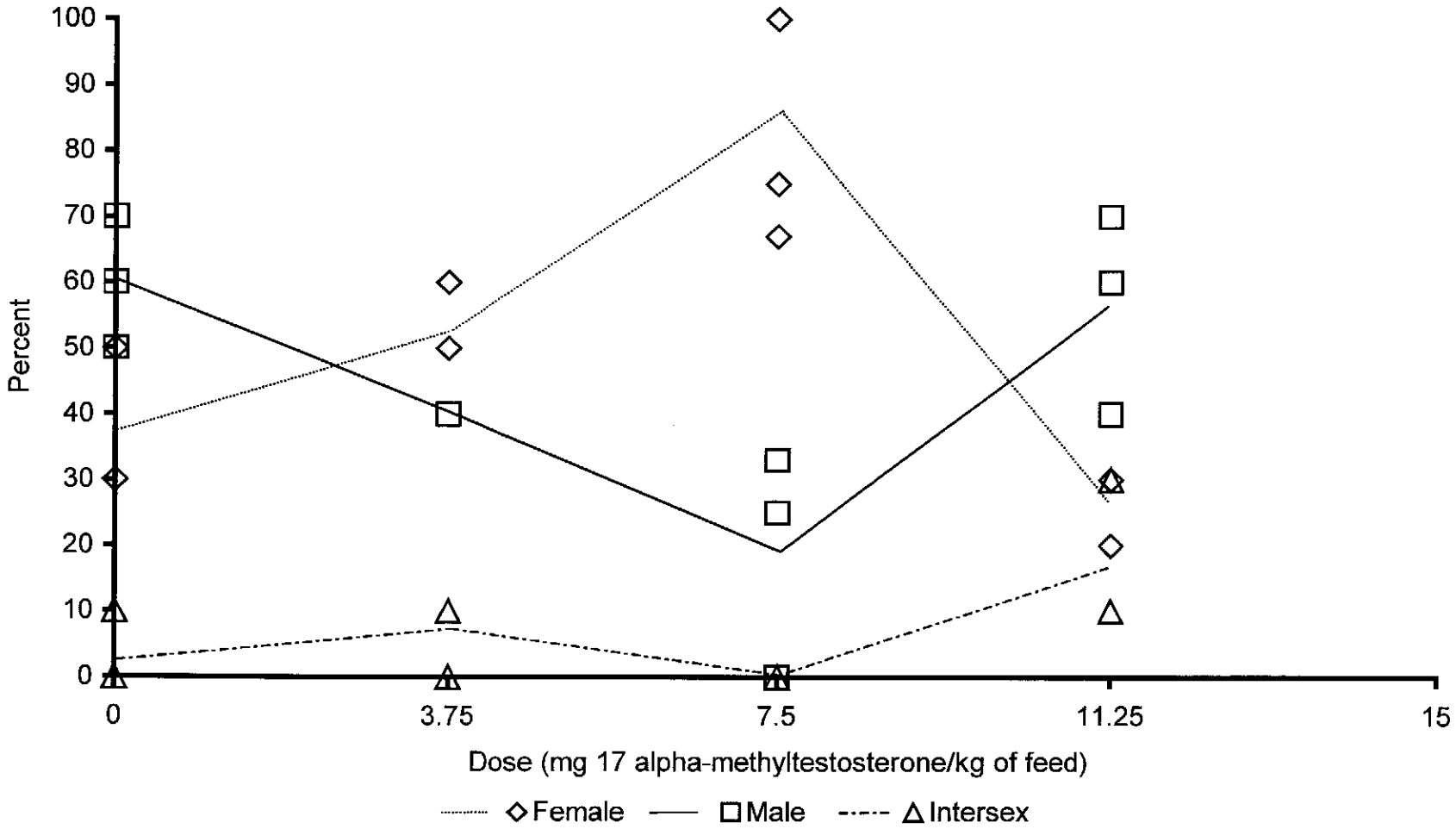

Figure 1. Mean distributions of males, females, and intersex muskellunge produced by feeding normal non-gynogenic muskellunge fingerlings a prepared diet fortified with three different dosages of 17α-methyltestosterone.

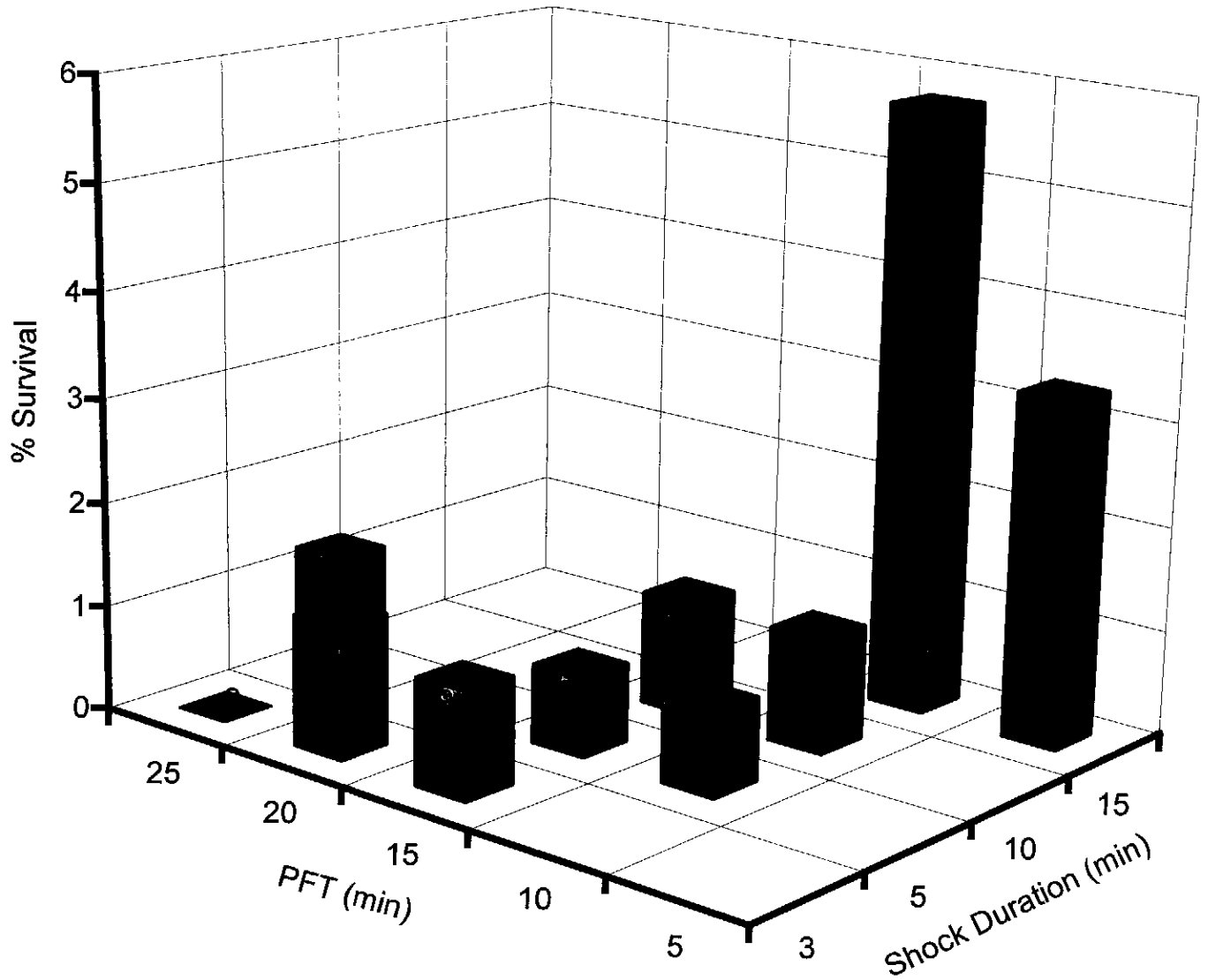

Figure 2. Mean actual survival of gynogens produced during entire project (data from segments 2,3 and 4) using pressure shocks of 7000 psi.. Numbers in bars indicate number of replicates at each treatment level. Ultraviolet irradiated (684 J/m^2) yellow perch spermatozoa were used to activate the muskellunge eggs. PFT = shock initiation time after fertilization. Shock Duration = total amount of time that pressure was applied.