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TRIAL HEAT SHOCKING TO INDUCE TRIPLOIDY
IN COHO SALMON, CHINOOK SALMON,
AND COHO X CHINOOK SALMON RECIPROCAL HYBRIDS

presented by

Douglas J. Sweet

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TRIAL HEAT SHOCKING TO INDUCE TRIPLOIDY IN COHO SALMON, CHINOOK SALMON, AND COHO X CHINOOK SALMON RECIPROCAL HYBRIDS

By

Douglas J. Sweet

A THESIS

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ABSTRACT

TRIAL HEAT SHOCKING TO INDUCE TRIPLOIDY
IN COHO SALMON, CHINOOK SALMON,
AND COHO X CHINOOK SALMON RECIPROCAL HYBRIDS

By

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The life span and growth capabilities of Pacific salmonids may be increased by sterilization via triploid induction. A heat shock, at 36°C, applied for one minute starting ten minutes after fertilization induced triploidy in 21% to 42% of the chinook salmon and between 0% and 14% chinook female x coho male salmon hybrids. No triploids were found in coho salmon or coho female x chinook male salmon hybrids.

Heat shocking significantly ($P \leq .001$) decreased survival by an average of 32% at swim up.

Chinook female x coho male salmon hybrids exhibited severe malformations in 30% of the individuals. Survival of this hybrid was 11% to 12% lower than pure chinook crosses.

Survival of coho female x chinook male hybrids was not significantly different from coho pure crosses.

This heat shock treatment was not optimal because triploid induction rates near 100% are desirable.

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INTRODUCTION

Salmonid aquaculture has benefitted from developments in nutrition, husbandry, and genetics. However, a major unsolved problem affecting production of Pacific salmonids is the reduced growth rate, flesh degradation, and increased mortality that occurs during maturation and spawning (Gjedrem, 1976; Lemoine and Smith, 1980; McBride and van Overbeeke, 1971; Refstie et al., 1977). An increase in production is expected if a method could be developed to inhibit the physiological changes associated with maturation. One potential solution is sterilization of fish intended for food or sport-fishery purposes.

Sterilization was previously tested on Pacific salmon in order to increase their life span. Kokanee salmon (Oncorhynchus nerka kennerlyi) were surgically castrated resulting in a longer life span (Robertson, 1961). Gonadectomy performed on sockeye salmon (Oncorhynchus nerka) also resulted in prolongation of life with cessation of the tissue degeneration that occurs during sexual maturation (van Overbeeke and McBride, 1971). The gonadal steroids, 11-ketotestosterone, 17α-methyltestosterone, and estradiol, are directly responsible for tissue degeneration during spawning and they also initiate hyperadrenocorticism causing further degeneration (Schreck and Fowler, 1982; van Overbeeke and McBride, 1971). Therefore, an effective

sterilization technique should prevent degeneration of tissue and mortalities associated with maturation in Pacific salmonids. Since fish have indeterminant growth, (Beverton and Holt, 1957) older sterile fish should continue growing without putting energy into reproduction.

Since surgical castration of large numbers of hatchery produced salmonids is not economical, other methods of sterilization have been tested. Steroid hormones were used to sterilize and alter the sex ratios of salmonids. This technique requires the eggs to be immersed in and the swim up fry fed on steroid hormones (Goetz, et al., 1979).

Another, more simplified method of sterilizing salmonids is the induction of triploidy. Triploidy, the condition of having three haploid chromosome sets (3N) instead of two haploid chromosome sets (2N), can be easily induced in various fish and amphibians. Triploidy was induced in various urodeles and anurans with a heat shock of 35.0°C - 37.0°C, applied to newly fertilized eggs for 4 to 7.5 minutes (Briggs, 1947). Briggs found the optimal time to administer the heat shock was 20 minutes post ferilization. This time corresponds to metaphase of the second meiotic division of the egg nucleus, which suggests that heat shock causes triploidy by preventing shortening of spindle fibers during anaphase. The spindle fibers are probably partially denatured by the heat (Briggs, 1947). Other researchers agree that triploid induction occurs through prevention of the second meiotic division. For example, marker

chromosomes were used to determine the origins of triploidy in the newt (Pleurodeles waltlii), and these observations proved that the two sets of maternal chromosomes and one set of paternal chromosomes that occured in a triploid individual arise from suppression of the second meiotic division of the egg (Ferrier and Jaylet, 1978).

Triploidy causes sterility in the adult organism because gametogenesis has been arrested in the gonial stages. Gametogenesis is probably arrested due to the odd chromosome number and resulting aneuploidy that occurs in the sex cells of triploids (Cassoni et al., 1984). In addition to possessing non-functional gametocytes, the gonads of triploids are often severely retarded in development. Gonads of triploid Atlantic salmon (Salmo salar) were reduced in size by 48% for males and 92.3% for females (Benfey and Sutterlin, 1984a). Gonad formation was also markedly reduced in triploids of carp, catfish, plaice, and plaice x flounder hybrids (Gervai, et al., 1980; Lincoln, 1981; Wolters et al., 1981b 1982d). Triploid salmonids, such as the rainbow trout (Salmo gairdneri), also show reduced gonad size and function but not to the degree as other species. The testes of male triploid rainbow trout develop normally except for nominal production of milt. Also steroid levels in triploid male rainbow trout did not differ significantly from diploids. However, female triploid rainbow trout had markedly reduced gonad size with low levels of gonadal steroids as compared to diploids (Lincoln and Scott,

1984; Thorgaard and Gall, 1979). By most indicators, triploid individuals are typically sterile.

There are exceptions to the rule of sterility in triploids because reproducing populations of triploid gynogenetic amphibians and fish have been discovered. All of these are specialized cases which frequently have unusual mechanisms for reproduction. For example, the silvery salamander (Ambystoma platineum) and the Tremblays salamander (Ambystoma tremblayi) are naturally occuring, all-female, triploid hybrids. These hybrids were the result of Jefferson salamanders (Ambystoma jeffersonianum) mating with blue-spotted salamanders (Ambystoma laterale). The silvery and Tremblays salamanders have no reductional meiotic divisions and sperm from one of the hybrid parental species males only activates the egg to develop (Behler and King, 1979). Triploid axolotl (Ambystoma mexicanum) females also produce offspring. No special meiotic mechanism to conserve normal chromosome numbers is involved because surviving offspring have variable chromosome numbers. Obviously the development of axolotls has some tolerance to aneuploidy (Frankhauser and Humphrey, 1950). A triploid fish species (Poeciliopsis sp.) also reproduces gynogenetically. In this case the triploid number of chromosomes is believed to be increased to hexaploid by an endomitotic division. The triploid number is then maintained by a meiotic division (Schultz, 1967). Due to the exceptions of sterility in these triploids, care must be taken when claiming any man-made triploids to be completely sterile.

Triploids usually do not differ from diploids morphologically. Triploid carp (Cyprinus carpio) were phenotypically identical to diploids except for a minor disturbance in scale pattern in the triploids (Gervai, et al., 1980). Comparisons of multiple morphological measurements, fin ray numbers and pharyngeal-teeth arrangement yielded no significant differences between diploid and triploid hybrid grass carp (Cassani et al. 1984). Triploid frog embryos and larvae develop normally and are identical to diploid larvae except for larger cell size and fewer cells in triploids (Briggs, 1947).

Cytologically, triploids can be distinguished from diploids. Karyological examination reveals triploids have an additional haploid set of chromosomes over the normal diploid set. The cell nuclei and cell size is usually larger for triploids than diploids. This occurs because triploids have 1/3 more DNA than diploids (Briggs, 1947).

Since triploids contain more DNA and have larger cells, it has been hypothesized that triploids also should grow faster and obtain larger sizes than diploids (Purdom, 1973). Growth in triploids of various species seems to be highly variable. The reported variation in triploid growth probably arises not only from actual differences between species but also the variation and inadequacies of experimental design. For example, triploid rainbow trout had slower growth than diploids as reported by Solar, et al., (1984), whereas Atlantic salmon had no significant differences in growth between diploids and triploids (Benfey and

Sutterlin, 1984a). Benfey and Sutterlin stated that although triploid Atlantic salmon may not grow faster than diploids in early growth stages, the triploids may outgrow the diploids during sexual maturation. Neither of the above studies included sexual maturation in their growth measurements. In other species, triploids were considered to grow faster and reach heavier weights compared to diploids. Triploid channel catfish (Ictalurus punctatus) were significantly heavier than diploids at 8 months old and older (Wolters et al., 1982d). This age and growth period corresponds with sexual maturity of the catfish. Grass carp x bighead carp hybrid triploids (Ctenopharyngodon idella ♀ x Hypothalmichthys nobilis ♂) grew faster than diploid hybrids (Cassani et al., 1984). Finally, triploid Tilapia aurea were larger than diploids at 14 weeks old (Valenti, 1975). The results of Valenti's study should be taken cautiously because of limited sample size. Only one to six polyploid fish per experimental group survived to the end of 14 weeks.

Another potential advantage of triploid induction is enhanced survival of triploid hybrids. Triploidy may increase survival of inter-generic or intra-generic hybrids by providing one complete maternal set of chromosomes. In an ordinary diploid hybrid, some vital hereditary material may be absent because only a haploid set of chromosomes originates from each parental species. In a triploid the extra set of maternal chromosomes gives the hybrid at least one complete diploid set of genes from one species. This compensates for any deficiencies caused by the

hybrid. For example Elinson and Briedis (1981) observed that diploid hybrids of bullfrog (Rana catesbiana) x green frog (Rana clamitans) died during gastrulation while the triploid hybrids flourished. Scheerer and Thorgaard (1983) induced triploidy in brown trout (Salmo trutta), brook trout (Salvelinus fontinalis), and rainbow trout hybrids. The triploid hybrids had higher survival rates than the diploid hybrids. In some cases the triploid hybrids had lower survival to the eyed stage of development but had better survival to the initiation of feeding. This reduced survival was probably due to effects of heat shock rather than triploidy.

Having discussed the advantages that inducing triploidy has in fish, the management implications for this process become clear. Triploid salmon and triploid hybrid salmon may benefit sport fisheries as well as aquaculture. Benefits for anglers include a potentially larger and different fish to capture, less flesh degradation during the spawning season, and in the case of hybrids, a combination of good traits such as faster growth with good fighting capability. Having combined characteristics, these triploid salmon hybrids may occupy a different ecological niche than their purebred parents. This offers the advantage of potentially increasing the carrying capacity and stability of the aquatic ecosystem involved. A good example of a successful hybrid occupying a new niche is the splake, a hybrid between lake trout (Salvelinus namaycush) and brook trout, that occupied a different niche than the lake trout and successfully avoided

heavy predation by the sea lamprey (Pillay and Dill, 1976). Triploid salmon can offer easy management of the number of fish stocked since no natural reproduction of these individuals should occur. Finally, sterile triploids may be introduced as exotic species to assess environmental impact before fertile fish are stocked.

Triploids and various polyploids have been produced and found to spontaneously occur in many salmonid species. Spontaneous triploids were discovered by Thorgaard and Gall, (1979), in the McCloud River rainbow trout strain. Utter et al. (1983) also found spontaneous triploids in pink salmon (Oncorhynchus gorbuscha).

Other polyploids have been induced by a variety of treatments with different species of salmonids. For example, the use of mitotic-inhibiting chemicals, such as cytochalasin B and colchicine, induced a variety of polyploids and chimeras (having a combination of diploid and polyploid cells) in rainbow trout (Refstie et al., 1977), atlantic salmon (Allen and Stanley, 1979), and brook trout (Smith and Lemoine, 1979). These chemical treatments, which were usually applied to the early embryo, did not lead to consistent results. Some individuals were diploid, triploid, tetraploid or mosaics combinations.

Cold shocking eggs and early embryos of salmonids have also been found to induce various polyploids and tetraploids. This technique has been used on brook trout, producing mosaic polyploids (Lemoine and Smith, 1980), and on Atlantic salmon

(Lincoln, et al., 1974). Tetraploids, which are theoretically fertile, are only desirable if they are crossed to diploids to produce all triploid offspring (Chourrout, 1984; Gjedrem, 1976).

The most effective means of inducing triploidy in salmonids was by heat shock. Using this method, triploids or tetraploids have been produced, with the specific result depending on the time after fertilization the eggs are shocked. Eggs shocked within the first hour of fertilization typically produce triploids, whereas eggs shocked about the time of the first mitotic division (about 5 hours post fertilization) typically produce tetraploids (Thorgaard et al., 1981). Heat shocks ranging from 26°C to 36°C, and lasting from 1 to 20 minutes, have been attributed to successful triploid induction (Benfey and Sutterlin, 1984b; Chourrout, 1980; Johnstone, 1985; Lincoln and Scott, 1983; Solar et al., 1984; Utter et al., 1983). Higher temperatures and longer heat shock periods increased the percentage of triploids but also decreased the number of survivors. The lower temperatures and shorter shocking periods increased fish survival but resulted in lower percentage of triploids. A review of the literature suggests the optimal temperature, time, and length of heat shock was between 26°C - 28°C, for 10-20 minutes, 20-25 minutes post fertilization (Solar et al., 1984). A heat shock of these parameters typically produced nearly 100% triploids and minimized heat shock mortalities. Utter et al. (1983) has previously induced triploidy in 58% to 84% of chinook salmon, pink salmon, and pink x chinook salmon reciprocal

hybrids. They used a ten minute heat shock, at 28°-30°C, applied ten minutes post fertilization.

The main objective of this research was to determine the feasability of inducing triploidy in coho salmon (Oncorhynchus kisutch), chinook salmon (O. tchawytscha), and coho salmon x chinook salmon reciprocal hybrids. Coho and chinook salmon were chosen because they are popular sport fish and are easily obtained from the Great Lakes. Hybridization is feasible because these species have overlapping spawning runs. The secondary objective of this research was to determine the viabililty of the hybrids, triploid hybrids, and triploid purebreds in relation to the pure breds, by recording survivorship to swim up. Previous reports of hybridization between these two species indicated poor survival of offspring from chinook females crossed to coho males and variable survival of offspring from coho females crossed to chinook males (Blanc and Chevassus, 1979, 1982; Chevassus 1979). Induction of triploidy in these hybrids was hoped to correct this situation and create a viable sport fish. The triploid hybrid would contain two haploid maternal sets of chromosomes (2N) and one haploid paternal set of chromosomes (1N). Hopefully this double set of maternal chromosomes, which is a complete chromosome set from the maternal species could compensate for any deficiencies of hereditary material caused by hybridization.

Although the most optimal heat-shocking technique seems to be at 26-28°C, for 10-20 minutes, at 20 to 25 minutes post fertilization, this technique was not used in this trial. The

majority of salmonids on which the optimal heat shocking technique was developed were smaller species, with smaller eggs, than the coho and chinook salmon. A hotter heat shock was thought necessary for adequate heating of the larger coho and chinook salmon eggs. Thorgaard et al. (1981) produced triploid rainbow trout at optimal numbers and survival with a heat shock of 36^{o}C for one minute, ten-minutes post fertilization. This hotter heat-shock regiment was chosen for this trial.

MATERIALS AND METHODS

Specific Crosses Tested

Eight different crosses, run in triplicate, were used to assess induction of triploidy in coho and chinook salmon. The crosses were:

1) coho ♀ x coho ♂
2) coho ♀ x coho ♂ + triploid induction
3) coho ♀ x chinook ♂
4) coho ♀ x chinook ♂ + triploid induction
5) chinook ♀ x chinook ♂
6) chinook ♀ x chinook ♂ + triploid induction
7) chinook ♀ x coho ♂
8) chinook ♀ x coho ♂ + triploid induction

Crosses 1 and 5 were used as references for egg and sperm quality for the coho salmon and chinook salmon, respectively in the experiment. Crosses 2 and 6 were to test triploid induction in coho salmon and chinook salmon, respectively, while crosses 3 and 7 were designed to test diploid reciprocal hybrids of coho and chinook salmon. Finally, crosses 4 and 8 were designed to test triploid induction in reciprocal hybrids between coho and chinook salmon.

Spawning Procedures

Eggs and milt of coho and chinook salmon were obtained from Michigan Department of Natural Resources, Fish Division, at the Little Manistee Weir on October 15, 1984. Complete hatchery procedures are outlined in Appendix A. It was estimated that approximately 500 eggs per test group would be needed to supply enough fish for karyotype samples (based on expected survival of chinook salmon in our fish laboratory, Garling and Masterson, 1985). To obtain 500 coho eggs per test group, the eggs of three females were pooled and mixed prior to separation into test groups and fertilization. The eggs of three chinook females were similarly treated. Milt from six coho males was pooled together because fully ripe coho males with adequate quantities of milt were difficult to obtain. Milt from only three chinook males was needed to supply adequate quantities for fertilization. Pooling of gametes would also minimize the effects of any sterile gamete donors.

Triploid Induction

To induce triploidy, the eggs were heat shocked in a 17-gallon fiberglass rectangular tank at ten minutes post fertilization. Water was circulated through this tank by an Autoflow external box filter. The water was heated to 36°C by two Fisher Automerse thermostatic immersion heaters. The eggs were suspended in this tank during heat shock in 2 mm-mesh floating screen boxes measuring 8" x 8" x 5". After application of the

heat treatment, eggs were immediately transferred to water at ambient temperature. All non-heat-shocked eggs were sham-heat shocked by moving and immersing the eggs for a similar period of time in water at ambient incubation temperature.

Egg Incubation Conditions

After all treatments, the eggs were allowed to water harden for 1 hour at ambient water temperature before transporting. The eggs were transported in plastic rectangular buckets, on ice, inside of coolers.

Upon arrival at MSU Fisheries Research Laboratory, the eggs were unpacked and numbers were estimated by the California Volumetric method (Leitritz and Lewis, 1980). Each test group was split into three equal replicates. Each replicate was randomly assigned a position in one of 24 vertical flow incubator trays. Each stack of eight trays was supplied with one gallon of aerated well water per minute at approximately 12°C.

To assess the various treatments, survival of eggs and fry was recorded weekly from fertilization to swim-up. Time within this developmental period was recorded as thermal units in degrees Celsius. Thermal units were calculated by summing the degrees Celsius that the incubation water was at for every successive 24 hour period. For example, if the water temperature was a constant 10°C for five days, then 50 thermal units would be accumulated. Eggs were considered dead when they turned white or fungused. Anti-fungal treatments were not used because frequent

egg picking prevented the spread of fungal infections. Care was also taken to prevent physical disruption of the eggs during the sensitive period between 48 hours post fertilization and eyeing approximately 25 days later (Leitritz and Lewis, 1980).

Statistical Analysis of Survival

Survival was compared statistically at two independent times during development. These times are considered crucial for salmon development and included the stages of (1) eyeing and (2) swim up. A three-way analysis of variance with orthogonal contrasts was used for these comparisons. The model is as follows:

$$Y_{ijkl} = u + \alpha_i + B_j + (\alpha B)_{ij} + Y_k + (\alpha Y)_{ik} + (BY)_{jk} + (\alpha BY)_{ijk} + E_{(ijk)l}$$

where u = mean survival

α_i = average effects of heat shock

B_j = average effects of maternal side of cross

$(\alpha B)_{(ij)}$ = interaction of α and B

Y_k = average effects of paternal side of cross

$(BY)_{jk}$ = interaction of B and Y

$(\alpha BY)_{ijk}$ = interaction of α, B, and Y

$E_{(ijk)l}$ = random effect of unspecified variables

(Gill, 1978).

Initial tests for homogeneous variance (f-max test) on survival for these times revealed heterogeneous variances. Three transformations were attempted on both time periods of data.

These transformations were:

1) $y = \text{Arcsin}(P)$

where P is proportion surviving at that time

2) $y = \log_e \frac{P}{1 - P}$

3) $y = \frac{1 - P}{P}$

(Gill, personal communication).

None of these transformations resulted in homogeneous variance ($P \leq .25$) for time (2) swim up and only transformation No. 2, above, achieved homogeneous variance for time (1) eyeing. Due to this heterogeneous variance, survival at time (1) eyeing was compared statistically after the data was transformed using transformation No. 2). Interactions and main effects were considered significant if a type I probability of error was .05 or less ($P \leq .05$).

Since time (2) swim-up data could not be successfully transformed to a homogeneous variance ($P \leq .25$), the transformation which most closely achieved this was used. Transformation No. 2 was used for this purpose but since it was not as complete as in time (1) eyeing the interactions and main effects were considered significant only if a type I error was .01 or less ($P \leq .01$) (Gill, personal communication).

Solid Tissue Karyotyping

To verify if heat treatment induced triploidy, karyotypes were performed on samples from each test group. Initially, karyotyping was attempted on swim up fry using Kligerman and Bloom's (1977) solid-tissue technique. Failure of this technique led to alternate trials with modifications on solid tissue karyotyping and some use of tissue culture. Modifications on the Kligerman and Bloom methods eventually resulted in moderately good results (See Appendix B). However, due to the amount of time the procedure required, the moderate success rate, and the age of the fish when sampling became possible, only twenty fish from each non-hybrid cross and ten fish from each hybrid group could be analyzed. These chromosome counts were completed when the fish were between four and six months old.

Metaphase chromosome spreads were examined with a Microstar American Optical Series One-Ten Laboratory Microscope. Slides made by the modified Kligerman and Bloom (1977) solid-tissue karyotyping technique were initially scanned at 100 power. Special attention was paid to the circumference of the dried-cell suspension rings. In this region the most acceptable metaphase spreads were found. Once a suspected spread was located at 100 power (a very small scattering of dots), a 1,000 power oil immersion lens was used while enumerating chromosomes. Chromosome contrast was optimal when stained with Giemsa and ammonium hydroxide intensifier; and when viewed with near maximum light power with diaphragms wide open. An eyepiece micrometer grid was

used as a position reference when counting chromosomes. This grid was centered over the metaphase spread while chromosome centromeres were counted in each grid square. The chromosomes within each grid square were counted in order starting with the upper left hand corner square, moving to each square to the immediate right, then down one row and back to the left. This pattern was followed as if each grid was counted as words that are read in this sentence. The actual count was performed on a Lion tally counter to minimize counting error and possible bias from prior knowledge of expected chromosome counts. Only three of the largest and most intact appearing metaphase spreads were counted per slide.

Graphs of Chromosome Counts

All metaphase spreads counted per test group were bar graphed according to chromosome count. These graphs essentially show the distribution of chromosome counts in the population of cells in that test group. Each individual fish is usually represented by three cells, or counts, on that graph. Expected diploid chromosome counts for chinook and coho salmon were taken from Simon (1963) and are indicated on the graphs. Calculated expected chromosome counts for diploid hybrids, triploid hybrids, and triploids are also indicated. The purpose of these graphs was to indicate if triploid cells are in the population. Hypothetically, if some triploids are present a bimodel distribution

peak is expected on the graph peaking at the expected diploid (dotted line) and triploid (dashed line) chromosome numbers.

Determination of Percentage of Triploid Individuals

Various parameters were set in order to determine the percentage of triploid individuals in each test group. This was deemed necessary because of the variable chromosome counts obtained and the likelihood that two overlapping partial diploid metaphase spreads may have appeared as a triploid. An individual fish was determined triploid if at least two of the three largest metaphase spreads had a chromosome count exceeding the median number between the expected diploid and triploid counts. This arbitrary limit was set in hopes of eliminating any artificially high diploid counts (due to counting error) but was low enough to include triploid spreads missing some chromosomes. Standard error for percent triploids was calculated for each test group by the binomial formula:

$$S.E. = \frac{wl(1 - wl)}{n}$$

where wl = percent triploid individuals

n = total number of fish examined per test group

(Gill, personal communication).

Karyograms

Photomicrographs of the best metaphase spreads in each test group were taken. An Olympus Photomicrographic System Camera, model PM-10-M with accompanying exposure meter, model EMM-7, was

used. Karyograms were made by projecting negatives with a Beseler enlarger onto a piece of paper. Chromosomes were traced, blackened in, and later cut out. The chromosome images were then arranged into karyograms according to a Denver type arrangement (Al-Sabti, 1985; Blaxhall, 1983a). This technique has the metacentric chromosomes arranged in order of decreasing size followed by acrocentric chromosomes also arranged in order of decreasing size. Due to similiarity in size and shape of many chromosomes exact homologous pairs might not have been placed together. Initially chromosomes were classified into four categories as described by Levan, et al., (1965). These four categories were determined by chromosome arm ratios and consisted of metacentric, submetacentric, subtelocentric and acrocentric chromosomes. This system was abandoned due to high variability of chromosome condensation and relatively poor resolution from the film (ASA 64) used for karyograms. A simplified two category technique for chromosome classification was used instead. Using this technique chromosomes were categorized as having the centromere position either near the center of the chromosome (metacentric-submetacentric) or near the ends of the chromosome (acrocentric-telocentric).

Erythrocyte Nuclear Preparations

In addition to karyotyping, erythrocyte preparations were made for each fish karyotyped. These preparations were taken as an alternate system for determining triploidy if karyotyping

proved inadequate. Erythrocyte cell and nuclear size can be used as an indicator of triploidy (Beck and Biggers, 1983; Benfey et al., 1984; Benfey and Sutterlin, 1984c; Cimino, 1973; Wolters et al., 1982c). Blood was taken in a heparinized capillary tube from the severed anterior dorsal aorta of fish being prepared for karyotyping. The blood was then expelled onto a clean slide, smeared with the edge of another slide, and allowed to air dry. Dried blood smears were fixed in methanol for two minutes, allowed to air dry again, and stained in buffered Giemsa as described in step 10 of Appendix B (Beck and Biggers, 1983).

Stained blood smears were observed under 1,000 X (using the same microscope for viewing metaphase spreads). Individual erythrocytes were randomly selected and nuclei width and length were measured using a micrometer scale. Any erythrocytes which appeared malformed, ruptured, or otherwise disrupted were not included in the measurements. Area and volume of the erythrocyte nuclei were calculated using formulae taken from Lou and Purdom, (1984). Area was calculated by the ellipse formula:

$$\text{Area} = (a)(b)\ /4$$

where a is the diameter of the short axis

b is the diameter of the long axis.

Volume was calculated by the formula:

$$\text{Volume} = \frac{a^2 b}{1.91}$$

Since karyotyping provided sufficient data, only a limited number of erythrocyte slides were measured. Erythrocytes from six chinook salmon determined by karyotyping to be triploid, and six chinook salmon determined by karyotyping to be diploid were fully analyzed. This small test sample was analyzed to check the validity of using erythrocyte dimensions as triploidy indicators for future work. Bar graphs were made displaying the distribution of triploid and diploid erythrocytes vs. length of long nuclear axis, length of short nuclear axis, area and volume of erythrocytic nuclei.

Disembryogenesis associated with the hybrid salmon was examined histopathologically and described by the Animal Health Diagnostic Laboratory at Michigan State University.

RESULTS AND DISCUSSION

Survival of Crosses

Incubation conditions for the eight different crosses can be considered favorable for optimal survival since survival of chinook salmon controls at time of swim-up was 91%. Garling and Masterson (1985), reported only 50% survival at swim up for chinook salmon incubated at the same facilities under similar conditions. Since survival of chinook salmon controls in this study was exceptional, it can be assumed that conditions were favorable for development of hybrid and triploid-induced (heat-shocked) crosses.

Three-way analysis of variance with orthogonal contrasts indicated that heat shocking was the main factor decreasing survival between fertilization to eyeing. Heat shocking decreased survival by an average of 24% (Figures 1 and 2). This difference is significant with a type I probability of error of .001 or less ($P \leq .001$). The majority of this decrease occurred in the few days post fertilization reflecting immediate effects from the heat shock.

All eggs originating from chinook salmon had better survival than eggs originating from coho salmon by time of eyeing. Mean

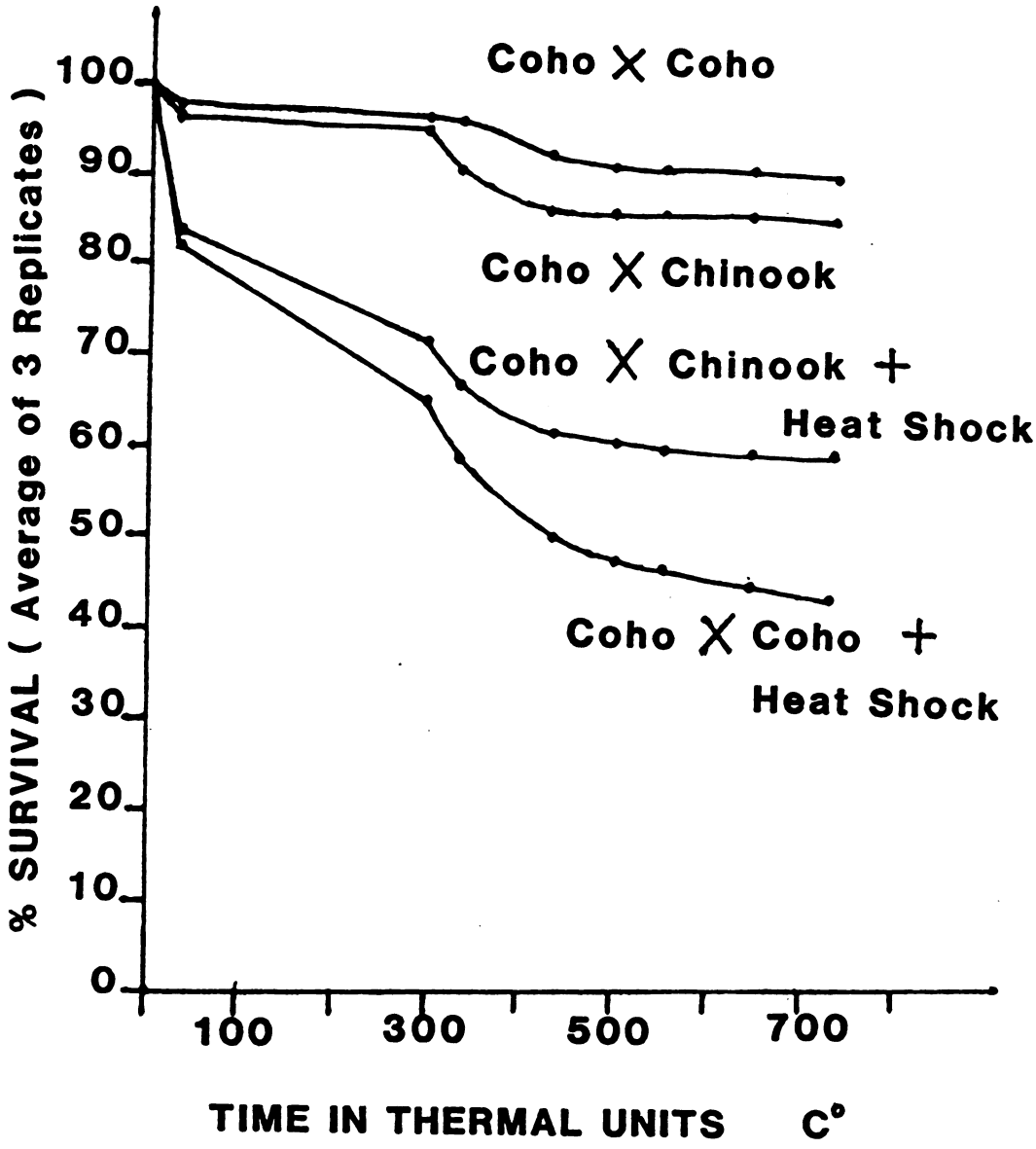


Figure 1. Survival of coho maternal side crosses from fertilization to swim-up. Eyeing occurred at approximately 285 thermal units C^{o}. Hatching occurred between 450 and 500 thermal units C^{o}.

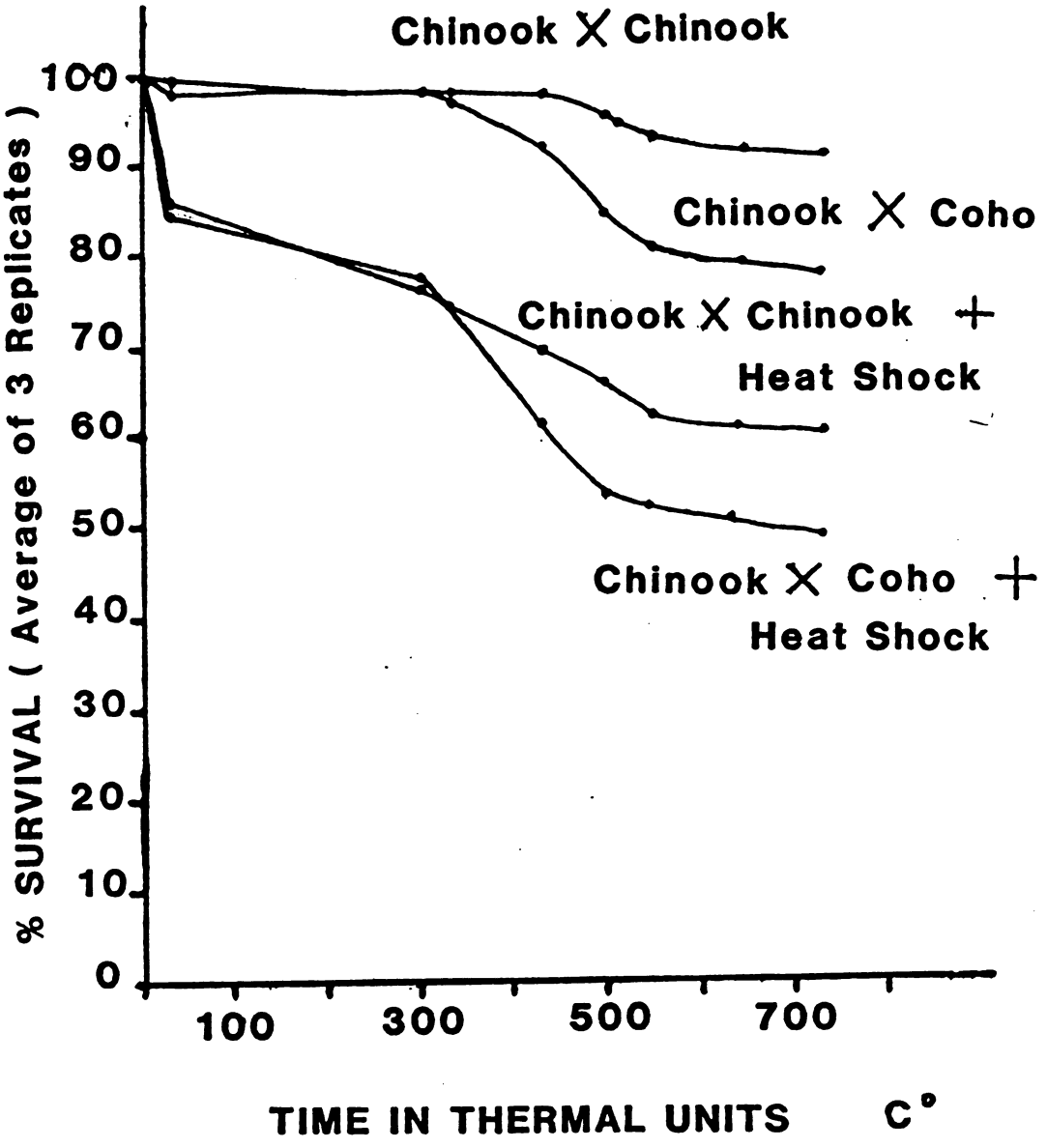


Figure 2. Survival of chinook maternal side crosses from fertilization to swim-up. Eyeing occurred at approximately 285 thermal units C^{o}. Hatching occurred between 500 and 550 thermal units C^{o}.

survival of chinook eggs was 7% higher than coho eggs and this maternal-effect was highly significant ($P \leq .001$).

At time of swim-up, heat shock was also found to significantly ($P \leq .001$) decrease survival by an average of 32%. A large proportion of these mortalities originated in the period prior to eyeing. This is evident from the similarity of survival curves after eyeing. The only difference between heat-shocked and non-heat-shocked curves is the displaced position of heat-shocked curves due to high mortalities prior to eyeing (Figures 1 and 2). This indicates, as would be expected, that heat shock was most damaging to development during the heat shock or in the stages immediately thereafter.

In contrast to significant maternal effects occurring between fertilization to eyeing, the period from fertilization to swim-up had significant main effects caused by the paternal source of the cross. During this period survival averaged 10% lower for eggs fertilized by coho males than for eggs fertilized by chinook males. This difference was significant with a type I probability of error of .001 or less ($P \leq .001$).

Hybrids between brown trout females and brook trout males were shown to have a similar pattern of maternal dominance of survival characteristics occurring before the eyeing stage (Blanc and Poisson, 1983). Paternal effects only become important after yolk sac absorption and in the case of many hybrids seems to be the source of blue sac disease (Blanc and Poisson, 1983). One potential explanation of the decreased survival of coho maternal

and paternal sides of the crosses is the observation that coho salmon were not at peak ripeness and peak of the spawning run when eggs and milt were taken. Ripe female and male cohos were difficult to obtain at egg-taking time, indicating a possible condition of underripe or overripe eggs. This could easily explain the lower survival of coho maternal-side crosses. Further evidence for the low quality of eggs obtained from coho salmon is seen in the sudden drop of survival of coho eggs within the first few days post fertilization (Figure 1).

Survival of all hybrid groups, except coho female x chinook male plus heat shock, was lower than their respective control groups at time of swim up. Survival of chinook female x coho male hybrids was 12-13% lower for both heat-shocked and non-heat-shocked groups as compared to pure chinook crosses. Survival of coho female x chinook male hybrids (84%) was lower but similar to survival of coho pure matings (87%). The exception to this trend was the higher survival of heat-shocked coho female x chinook male hybrids (61%) than heat-shocked coho pure matings (43%). This extremely low survival of heat-shocked coho pure matings may be explained by the compounded effects of low survival characteristics of coho eggs, the low survival characteristics occurring from coho paternal side crosses, and low survival caused by the heat-shocked treatment. Lower survival of hybrids is expected. Lower survival of heat-shocked hybrids over diploid hybrids contradicts what other researchers have found and what is expected of triploids. Using female brook trout x male brown trout and

female rainbow trout x male brown trout Scheerer and Thorgaard (1983) listed a hierarchy of survival. Diploids of either species had the highest survival, followed by triploids, triploid hybrids, and diploid hybrids having the lowest survival. In this case triploid hybrids had lower survival to eyeing (probably from detrimental effects of heat shock) but better survival to initiation of feeding. Induced triploidy also increased the survival of Tilapia nilotica x Tilapia aurea hybrids (Chourrout and Itskovich, 1982). Heat shocked hybrids may not have better survival in this research because of relatively low triploid induction rates of this specific heat-shock treatment. Coho female x chinook male hybrid heat-shocked groups contained no triploids. Heat-shocked chinook female x coho male hybrid groups contained 0 to 14.3% triploids (Table 1). This low number of triploids may not have been adequate to overcome the negative survival stress of hybridization and heat shocking. If triploid induction was more successful and in the range of at least 80-90% of the individuals, survival might have been considerably higher.

Disembryogenesis of Hybrid Offspring

Interesting cases of disembryogenesis were observed in hybrid offspring of chinook and coho salmon. Marked malformations of chinook female x coho male hybrids was noted shortly after hatching. These malformations were present in as many as 30% of the hybrid individuals at any given time. The gross appearance of these fish suggested a degeneration of the cervical

and caudal areas (Figures 3 and 4). Scoliosis, lordosis, and sometimes both were present. Fish continued to developed these traits over a four month period of time, with cervical degeneration appearing first, followed by caudal degeneration and discoloration. The degeneration made swimming and feeding difficult for the fish, eventually causing death.

A very small percentage (less than 1%) of fish in the other types of crosses also developed these problems. Specifically, coho female x chinook male hybrids had a few individuals with slight degeneration located only in the cervical area and no degeneration in the caudal areas. Some of these fish also had shortened gill opercula.

Normal and malformed individuals at four weeks post hatching were sacrificed, preserved, and sent to the Animal Health Diagnostic Laboratory at Michigan State University for diagnosis. Radiographic and histopathological examinations revealed the following results. Vertebrae were poorly ossified and malformed into triangular shapes. Areas of deformed vertebrae coincided with scoliosis and lordosis. In areas where degeneration occurred there was unequal development of segmental myotomes with muscle tissue missing in certain areas and loosely organized in other areas. Epithelium in the degenerated areas was lacking scales but was pigmented and contained mucus glands. No inflammatory cells were located in these areas indicating a non-infectious etiology. The final diagnosis from the Animal Health Laboratory was musculo-skeletal disembryogenesis of unknown

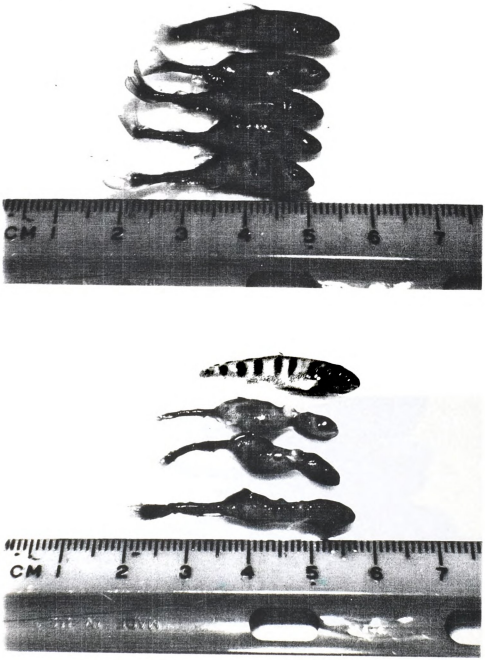
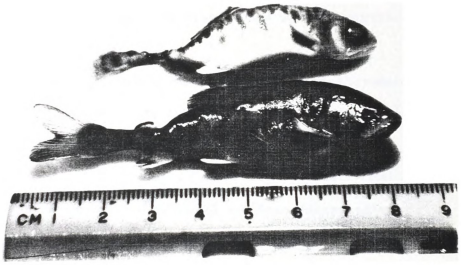


Figure 3. Varying degrees of disembryogenesis occurring in swim-up chinook female x coho male hybrid salmon. Most severe on bottom with decreasing disembryogenesis toward top. A normal hybrid is in each photo (top) for comparison.



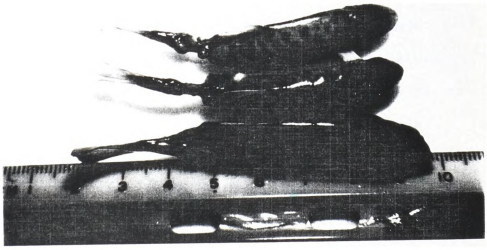


Figure 4. Four month old chinook female x coho male hybrid salmon showing moderate disembryogenesis (Above - side view, below, top view).

origin (Figures 3 through 7 for comparisons of normal and abnormal individuals). A genetic cause may be suggested since only chinook female x coho male hybrids had disembryogenesis in large percentages.

Other workers have found various forms of disembryogenesis or "cripples" associated with hybrids. Hybrids between Atlantic salmon and pink salmon produced non-viable fry with severe morphological anomalies. Some of these fry apparently had only one eye directed forward on the head and were described as "cyclops like" (Loginova and Krasnoperova, 1982). Simon and Noble, (1968) also reported variable survival of reciprocal crosses of pink salmon and chum salmon (Oncorhynchus keta). Chum salmon males x pink salmon females produced relatively normal appearing offspring with few mortalities. The reciprocal cross, with chum salmon females x pink salmon males suffered total mortalities from weak deformed offspring. All hybrids between chum and pink salmon had less robust caudal peduncles similar to the results in chinook female x coho male hybrids from this study. In brown trout female x brook trout male hybrids, the percentage of cripples depended on which individual female brown trout was used in the cross (Buss and Wright, 1956). This occurrence may also hold true for this study. There is the possibility that one of the three chinook females used in the chinook female x coho male cross was genetically responsible for the 30% disembryogenesis observed. Buss and Wright, (1956) also reported high variation in viability of fry in splake x brook

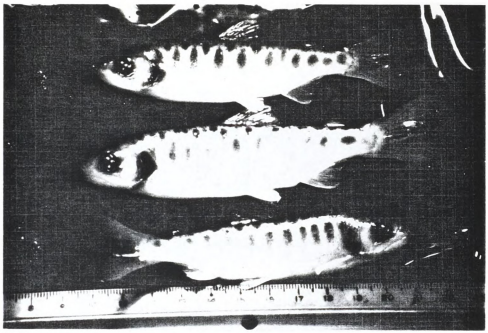


Figure 5. Six month old coho female x chinook male hybrid salmon.

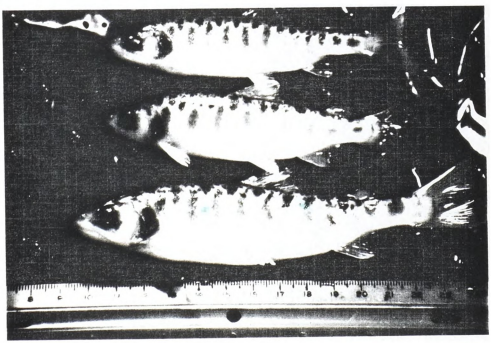


Figure 6. Six month old chinook female x coho male hybrid salmon.

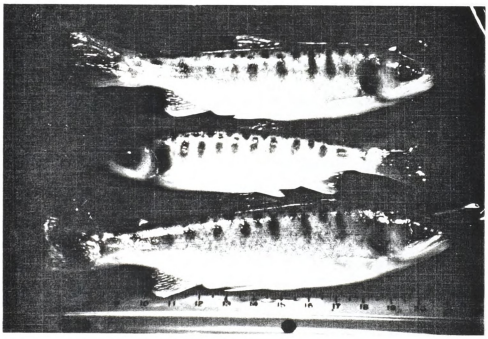


Figure 7. Six month old coho salmon.

trout hybrids and high percentages of "caudal cripples" in brook trout females x lake trout males.

Previous trials of chinook salmon x coho salmon hybrids had mixed results. Coho females x chinook males were rated as a potentially useful hybrid (Chevassus, 1979 and Refstie et al., 1982). This hybrid was shown to have a hatching rate of 40-88% of control. (Chevassus, 1979 and Refstie et al., 1982), and the growth rate of this hybrid was reported to be five times the control rate at 230 days of age (Refstie et al., 1982). My study agrees with these findings since coho female x chinook males had minimal disembryogenesis with survival slightly less then control at time of swim up (84% compared to 87%). Chevassus (1979) reported lower success for chinook females x coho males because only a few individuals survived to one year old. My study again agrees with these findings since this cross contained severe disembryogenesis and lower survival compared to control (12-13% lower). Blanc and Chevassus (1979 and 1982) also reported good survival of coho female x chinook male hybrids. Their study reported survival as high as 50% of control up to the fourth month post hatching. These hybrids were noted to have slower growth and achieved a smaller size compared to coho controls. In contrast, using Foerster's (1935) work as an example, Chevassus (1979), stated that hybrids using coho salmon were typically non-viable. Specifically, coho females x chinook males did not even survive to the eyed stage.

The cause of non-viability in hybrids is often difficult to ascertain. Blue sac disease (i.e. the inability to absorb the yolk sac properly), has been implicated in many hybrid problems (Blanc and Poisson, 1983; Buss and Wright, 1956). Other potential explanations for hybrid mortalities include failure at fertilization or embryogenesis, failure at hatching, differences in incubation times, egg sizes, or chromosome counts causing incompatibilities between the two species (Buss and Wright, 1956). Crippling and disembryogenesis is obviously responsible for many mortalities and is probably caused by some genetic inbalance of the hybrid genome during hybrid development. In the present study the majority of hybrid mortality occurred between eyeing and hatching (Figure 1 and 2). In fact, many chinook female x coho male hybrid alevins were observed to have died in the process of hatching. Disembryogenesis definitely contributed to mortalities of the hybrids, especially after the period of swim up when these individuals were not able to swim normally and compete for feed. In Atlantic salmon, high mortalities at time of hatching have been associated with defective enzymes (Battle, 1944). Hybrids with defective hatching enzymes would have problems during hatching, whereas hybrids with severe disembryogenesis may not have the physical capability to hatch. It would be interesting to discern what genetic and developmental mechanism is disrupted to cause the disembryogenesis in hybrid salmonids.

Maternal-Dominance in Hybrids

Another interesting phenomenon observed in coho x chinook salmon hybrids was maternal dominance in the characteristics of the dorsal and anal fin. All hybrids originating from coho eggs retained the coho salmon's elongated and white colored leading fin rays of the anal and dorsal fin. In contrast, all hybrids originating from chinook eggs lacked this white leading edge and retained the appearance of chinook salmon. Other authors have reported various forms of maternal and paternal dominance in fish species. Hybrids between chum salmon males and pink salmon females may show characteristics of one parent, the other parent, unlike both parents, or a combination from both parents (Simon and Noble, 1968). The F_1 generation of chum salmon females x pink salmon males developed a dorsal hump which was intermediate in size to the large hump observed on pink salmon males. Tail spots, which are very evident on pink salmon, are present but are reduced in size on the hybrid. Another characteristic which is expressed in the hybrid in an intermediate manner is tooth size. Pink salmon have small teeth while chum salmon have large teeth. The hybrids have intermediate sized teeth. In four different characteristics the hybrid chum x pink salmon resembles either one parent species or the other. The pink salmon's white belly and white background coloration is retained over the dark belly and yellow background coloration of the chum salmon. Parr marks of the chum salmon, which are absent in pink salmon, remain evident in the hybrid. Gill raker counts in the hybrid are

closer to pink salmon counts than to chum salmon counts. Finally, scale counts of the first row of scales above the lateral line resembles chum salmon scale counts more closely than pink salmon scale counts. Simon and Noble, (1968) explain the parr mark characteristics as a simple Mendelian recessive. The other characteristics are considered to be inherited in a multiple gene system.

Reciprocal hybrids between channel catfish (Ictalurus punctatus) and blue catfish (Ictalurus furcatus) exhibited paternal predominance (Dunham, et al., 1982). The male parent of these two hybrids was more responsible for determining the external appearance, swim bladder shape, and fin ray number of the hybrid offspring. In addition, growth and morphometric uniformity also exhibited paternal predominance. Finally, even behavior was effected by paternal predominance. Susceptibility to capture by seine was influenced by the male parent more than the female parent (Dunham, et al., 1982).

Gynogenesis Versus Hybrid Production

Maternal dominance seen in the coho salmon x chinook salmon hybrids actually may be an indicator of gynogenetic production of the maternal species. Gynogenesis has been induced in various salmonids with several treatments. Gynogenetic rainbow trout were produced by fertilization with irradiated sperm coupled with heat shock (Chourout and Quillet, 1982; Purdom et al., 1985). Gynogenesis has also occurred in rainbow trout through the use of

pressure shocks of 7,000 psi applied for 4 minutes, 40 minutes post fertilization in conjunction with irradiated sperm (Chourrout, 1984). Finally, cold shock was also used to induce gynogenesis in coho salmon (Refstie et al., 1982). In my study, gynogenetic production may have occurred in the hybrids by activation of the maternal genome by sperm from the paternal species. The sperm would contribute no genetic material to the zygote. Most offspring activated in this matter would be haploid and probably would be inviable (Refstie et al., 1982). However, if fertilization via a hybrid sperm was associated with a disruption of the second meiotic division of the egg, then a diploid gynogenetic may have been produced. A diploid gynogenetic is produced if the sperm pro-nucleus is excluded from fusion, while a triploid hybrid is produced if the sperm pronucleus is included (Chevassus, 1983). Spontaneous prevention of the second meiotic division by fertilization with another species sperm has occurred in grass carp females crossed to big head carp males. In this case retention of the sperm pronucleus creates a triploid hybrid (Allen and Stanley, 1983; Cassani, et al., 1984).

If gynogenesis occurred with the coho salmon x chinook salmon hybrids then the similar appearance of the offspring to the maternal species could not be contributed to maternal dominance. These offspring would appear identical to the maternal side of the cross because they are gynogenetics of the maternal side. The possibility of such an occurrence is increased dramatically in the heat shocked hybrid groups because

the heat shock applied to induce triploidy may also induce diploid gynogenesis if the sperm pro-nucleus fails to unite with the egg pro-nucleus. Care must be taken when a hybrid is claimed to be produced because of the above mentioned possibilities. These pseudo-hybrids (gynogenetics) may be differentiated from true hybrids by morphological characteristics, physiological characteristics, biochemical characteristics, or karyological characteristics (Chevassus, 1983). Since hybrids do not always have intermediate characteristics of the two species involved, morphological, physiological, and biochemical characteristics are not always reliable at determining validity of a hybrid's genomes. The study of karyotypes is the only reliable means of determining the validity of an individual's genetic structure (Chevassus, 1983). Gynogenesis was not found in my study but before I discuss the evidence for this the various characteristics of karyograms from coho and chinook salmon must be discussed.

Karyograms of Coho Salmon

Chromosome counts of all metaphase spreads from coho salmon indicated a peak occurrance and probable diploid number of 60 (Figure 8). This number agrees with the findings of Gold et al. (1980) and Simon (1963). Karyograms of coho salmon indicate a range of 44 to 46 metacentrics-submetacentrics and 14 to 16 acrocentric-telocentric chromosomes (Figures 9 and 10). Simon (1963) describes coho salmon having 52 metacentrics-submetacentrics and

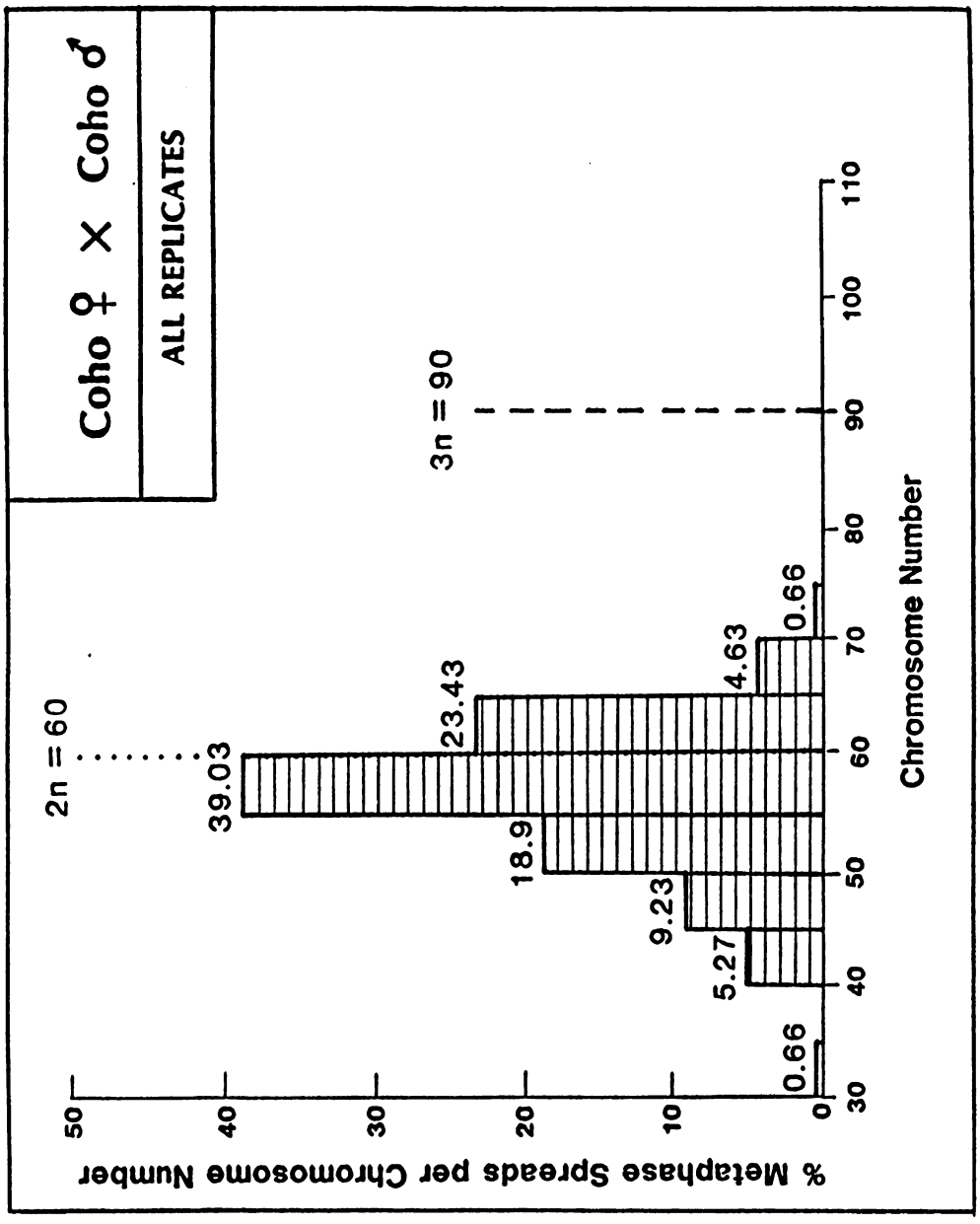


Figure 8. Chromosome number composition of metaphase spreads sampled from coho salmon.

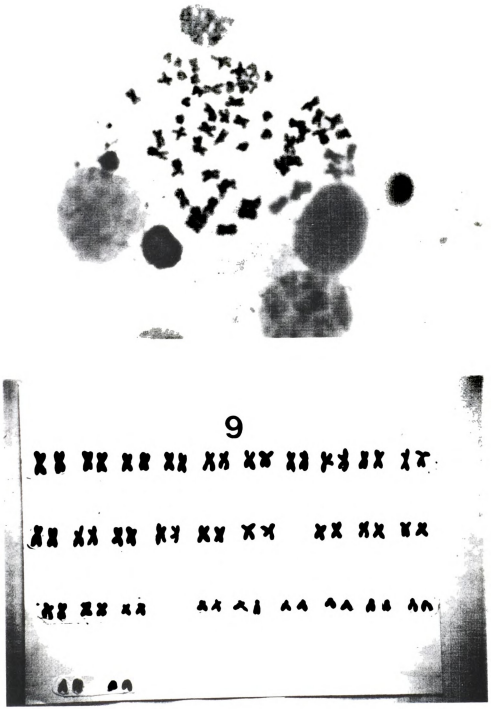


Figure 9. Metaphase spread (above) and karyogram (below) of coho salmon diploid with 60 chromosomes (44 metacentric-submetacentrics and 16 acrocentric-telocentrics).

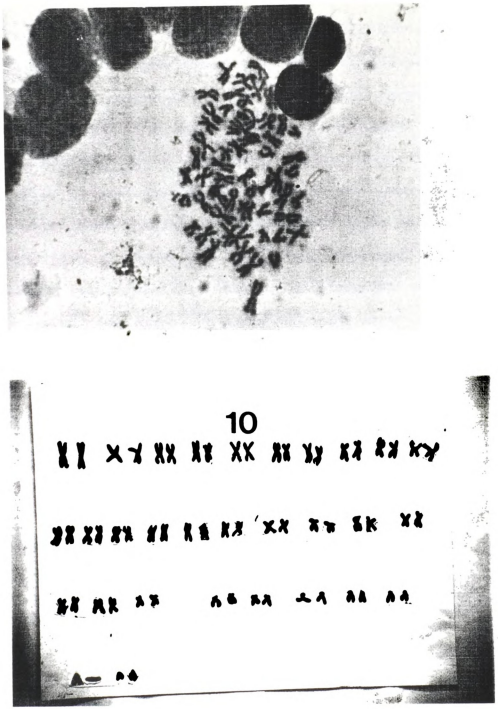


Figure 10. Metaphase spread (above) and karyogram (below) of coho salmon diploid with 60 chromosomes (46 metacentric-submetacentrics, and 14 acrocentric-telocentrics).

eight acrocentrics. This dissimiliarity reflects differing nomenclature systems rather than differences in chromosome morphology. He observed and made drawings from chromosomes in anaphase in contrast to my use of metaphase chromosomes. Simon's drawings, made from camera lucida and direct microscope observations, appear to have much more detail than my own, hence, his nomenclature system is different. Simon breaks up the chromosome morphology into two groups. The first group consists of V-shaped (metacentric) and J-shaped (submetacentric) chromosomes grouped together. The second group consists of only rod shaped (acrocentric) chromosomes. Chromosomes in my study were grouped into the acrocentric-telocentric category when the centromeres were at the end, or nearer to the end of than the middle of the chromosome. In contrast, because of finer resolution, Simon (1963) only categorized chromosomes as acrocentric when the centromere was located at the end of the chromosome. When the nomenclature system employed in this study is used on Simon's coho salmon karyogram, 46 meta-centric-submetacentrics with 14 acrocentric-telocentric chromosomes is observed. This completely agrees with the findings from karyograms in my study.

Karyograms of Chinook Salmon

Chromosome counts for chinook salmon metaphase spreads peaked at and indicate a probable diploid number of 68 (Figure 11). A diploid number of 68 chromosomes for chinook salmon agrees with the findings of Gold et al., (1980) and Simon (1963).

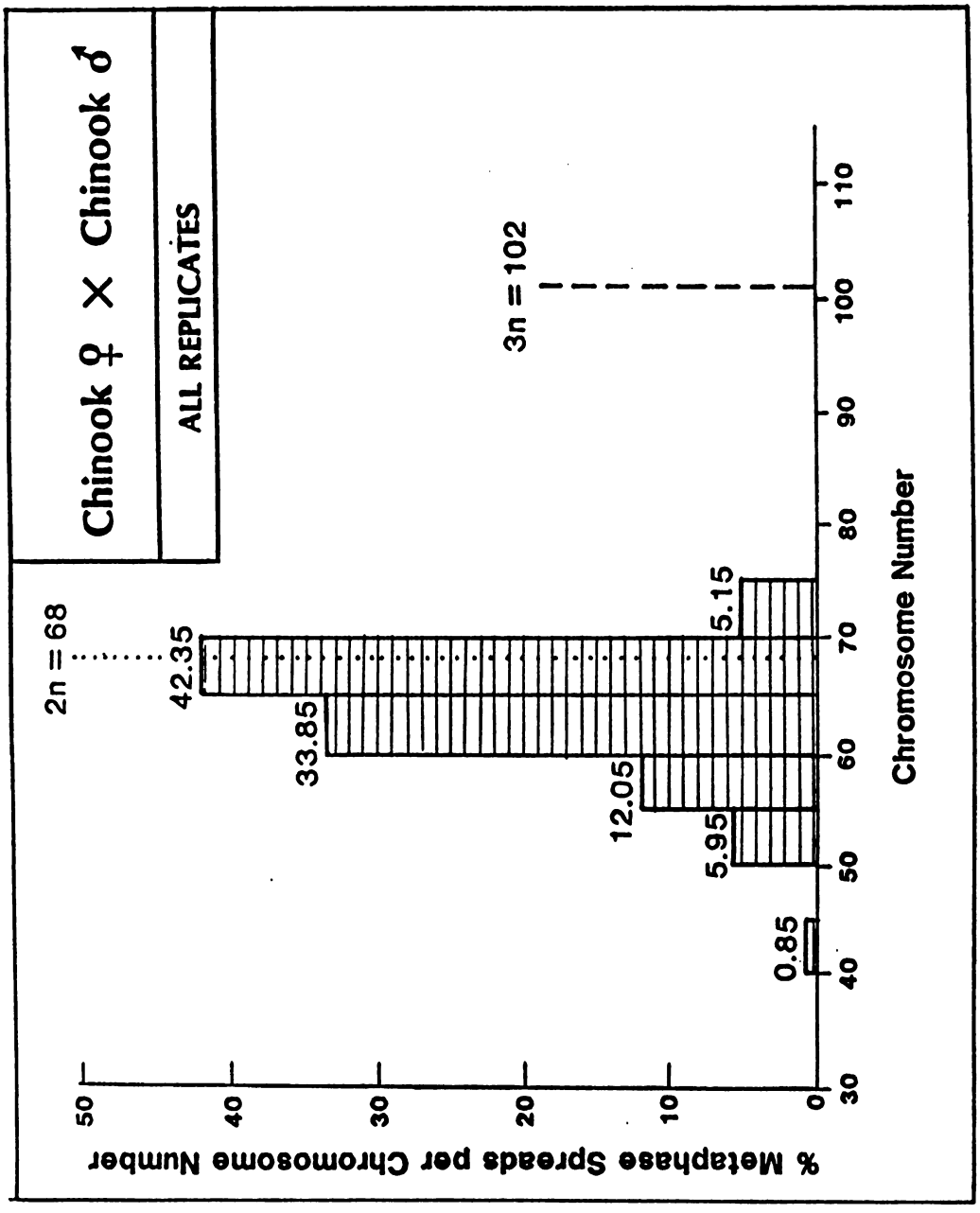


Figure 11. Chromosome number composition of metaphase spreads sampled from chinook salmon.

Simon (1963) also classified the chinook's chromosomes as 36 metacentric-submetacentric with 32 acrocentrics. This is similar to the findings of my study where 34 to 36 metacentric-submetacentrics and 32 to 34 acrocentric-telocentrics were observed (Figures 12 and 13).

Verification of Hybridization: Karyograms of Reciprocal Hybrids Between Coho and Chinook Salmon

Since standard chromosome complements of chinook and coho salmon are known, the expected hybrid complement may be determined. This expected complement may then be compared with the actual hybrid karyograms to determine if true hybridization or if gynogenetic production has occurred. Taking the haploid chromosome number for chinook salmon (34) and adding it to the haploid chromosome number of coho salmon (30) results in an expected diploid hybrid number of 64. The peak chromosome number found in chinook female x coho male and coho female x chinook male bar graphs was 64 (Figure 14 and 15). This indicates that true hybridization, rather than gynogenetic production, occurred. The peak chromosome number would have been equal to the maternal diploid number if gynogenetic production had occurred. Further evidence for true hybridization can be found upon examination of hybrid karyograms. Since chinook salmon have 34 to 36 metacentric-submetacentrics and 32 to 34 acrocentric-telocentrics, then a haploid number of 17 to 18 metacentric-submetacentrics and 16 to 17 acrocentric-telocentrics would be expected to be

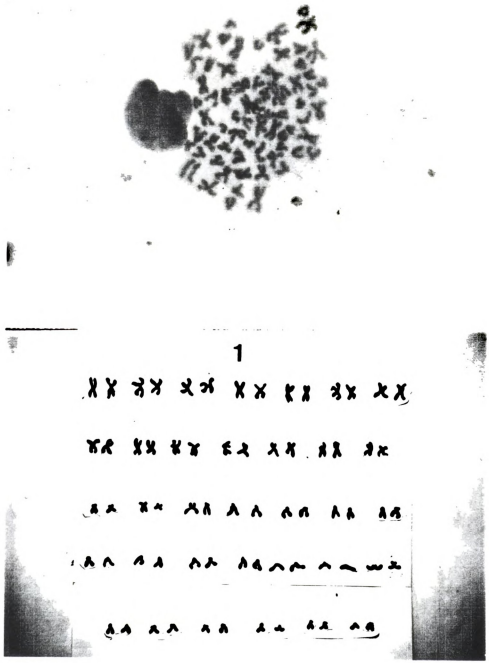


Figure 12. Metaphase spread (above) and karyograms (below) of chinook salmon diploid with 68 chromosomes (34 metacentric-submetacentrics and 34 acrocentric-telocentrics).

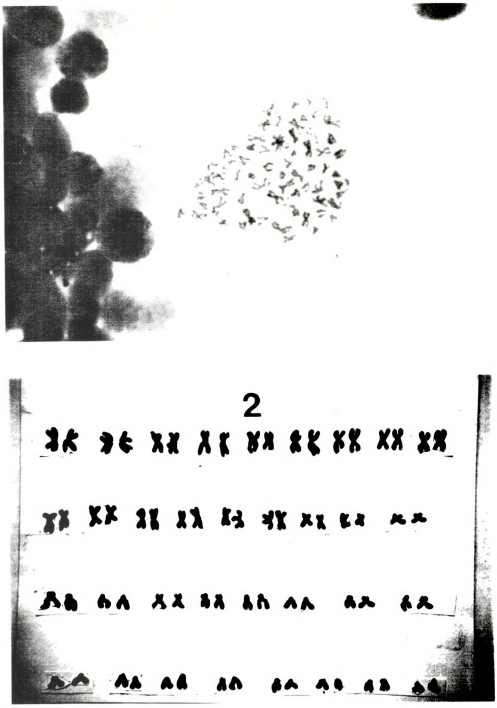


Figure 13. Metaphase spread (above) and karyogram (below) of chinook salmon diploid with 68 chromosomes (36 metacentric-submetacentrics and 32 acrocentric-telocentrics).

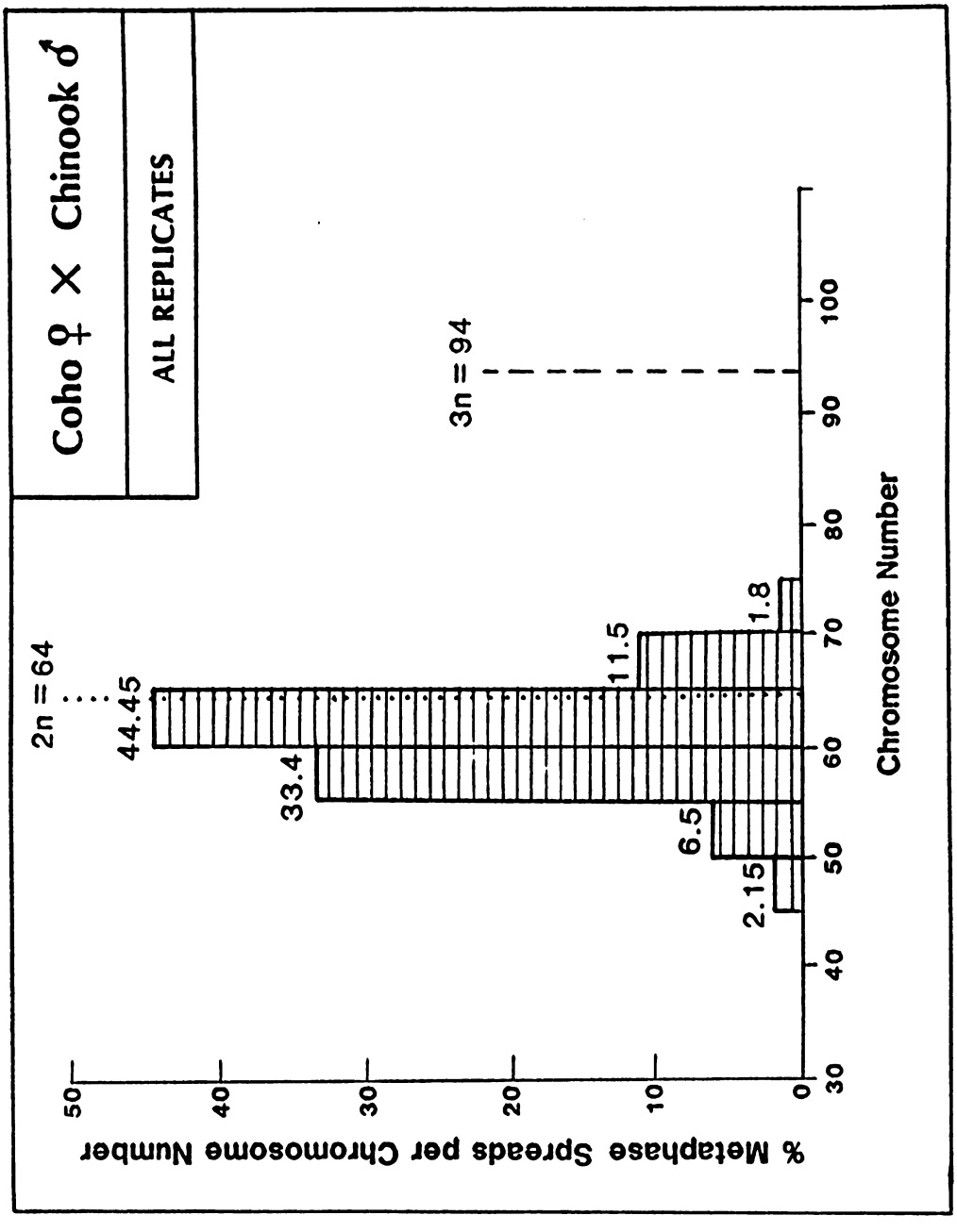


Figure 14. Chromosome number composition of metaphase spreads sampled from coho female x chinook male hybrid salmon.

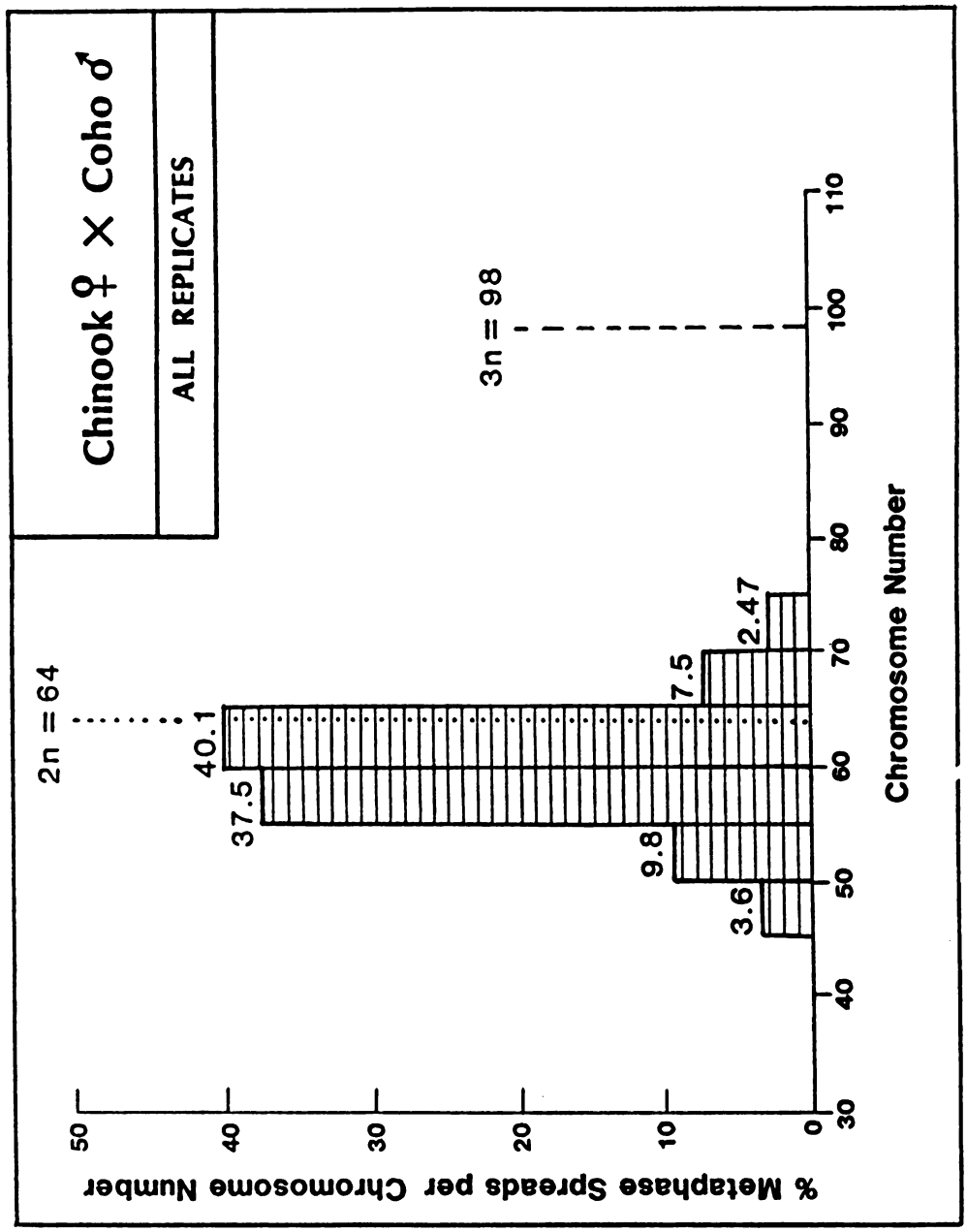


Figure 15. Chromosome number composition of metaphase spreads sampled from chinook female x coho male hybrid salmon.

contributed to the hybrid. Likewise, coho salmon were determined to have 44 to 46 metacentric-submetacentrics and 14 to 16 acrocentric-telocentrics. The coho salmon's haploid contribution to the hybrid would be 22 to 23 metacentric-submetacentrics and 7 to 8 acrocentric-telocentrics. Combining the chinook and coho haploid chromosome complements gives an expected hybrid with 39 to 41 metacentric-submetacentric and 23 to 25 acrocentric-telocentric chromosomes which was observed in hybrid karyograms shown in Figures 16 through 19. Figures 16 and 18 show karyograms of individuals with 40 metacentric-submetacentric and 24 acrocentric-telocentric chromosomes. Figure 17 shows the karyogram of an individual with 40 metacentric-submetacentric and 23 acrocentric-telocentric chromosomes. Figure 19 shows an individual with 40 metacentric-submetacentric and 25 acrocentric-telocentric chromosomes. Figure 20 shows the karyogram of an individual that deviates the furthest from the expected hybrid number with 38 metacentric-submetacentric and 27 acrocentric-telocentric chromosomes. This last example is much closer to an expected hybrid karyogram than either parental species karyogram indicating it is a true hybrid. This is true because when the differences in chromosome number between the deviant hybrid and coho salmon, the deviant hybrid and chinook salmon, and the deviant hybrid and expected hybrid are compared; the difference is least between the deviant hybrid and the expected hybrid chromosome counts. Specifically, the deviant hybrid has two to four extra metacentric-submetacentric chromosomes and five to

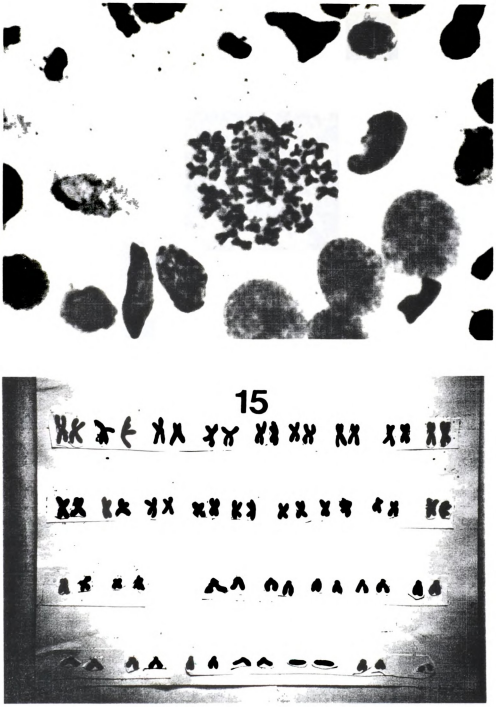


Figure 16. Metaphase spread (above) and karyogram (below) of coho female x chinook male hybrid diploid salmon with 64 chromosomes (40 metacentric-submetacentrics and 24 acrocentric-telocentrics).

Figure 17. Metaphase spread (above) and karyogram (below) of coho female x chinook male hybrid diploid salmon with 63 chromosomes (40 metacentric-sub-metacentric and 23 acrocentric-telocentrics).

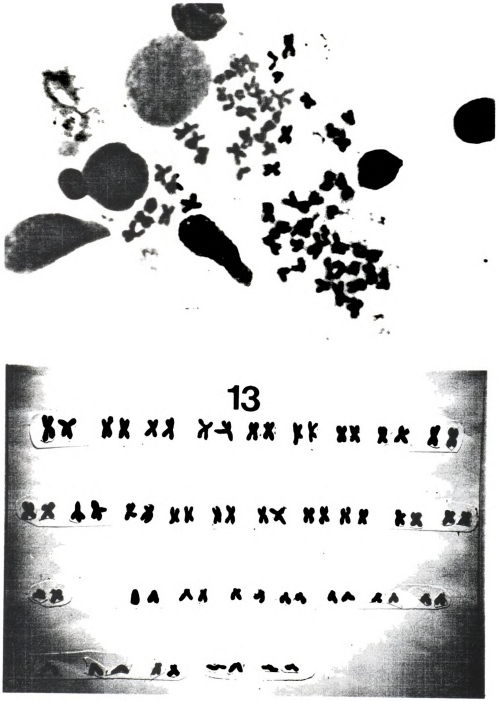


Figure 18. Metaphase spread (above) and karyogram (below) of chinook female x coho male hybrid diploid salmon with 64 chromosomes (40 metacentric-submetacentrics and 24 acrocentric-telocentrics).

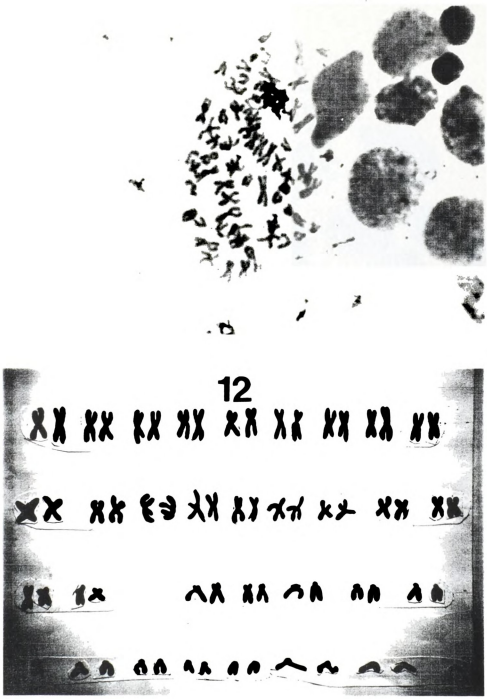


Figure 19. Metaphase spread (above) and karyogram (below) of chinook female x coho male hybrid diploid salmon with 65 chromosomes (40 metacentric-submetacentrics and 25 acrocentric-telocentrics).

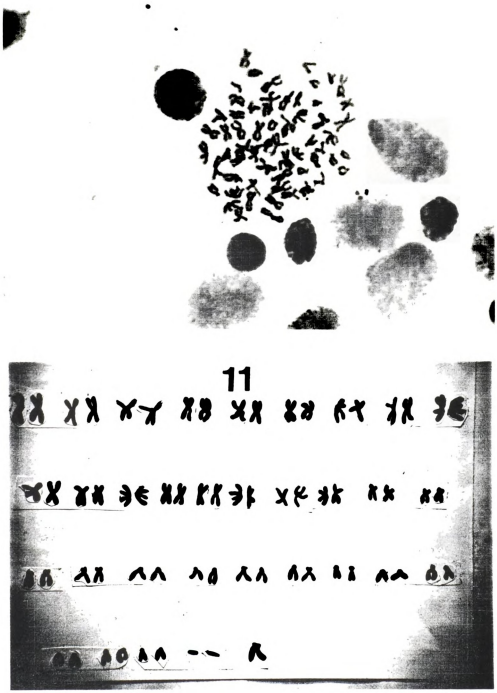


Figure 20. Metaphase spread (above) and karyogram (below) of chinook female x coho male hybrid diploid salmon with 65 chromosomes (38 metacentric-submetacentrics and 27 acrocentric-telocentrics).

seven fewer acrocentric-telocentric chromosomes when compared to the chinook salmon karyograms. The deviant hybrid also has six to eight fewer metacentric-submetacentric chromosomes and 11-13 extra acrocentric-telocentric chromosomes when compared to coho salmon karyograms. The smallest difference occurs when the deviant hybrid is compared to the expected hybrid chromosome counts. The deviant hybrid has only one to three fewer metacentric-submetacentric chromosomes and two to four extra acrocentric-telocentric chromosomes when compared to the expected hybrid counts.

Karyograms of Triploid Chinook Salmon

Karyogram and metaphase spread chromosome count distributions also indicate triploidy was induced in heat-shocked chinook salmon and chinook female x coho male salmon hybrids. Bimodal peaks centered around the expected chinook salmon diploid (2N = 68) and triploid (3N = 102) number indicate triploid and diploid individuals were contained within the heat-shocked chinook salmon pure crosses (Figure 21). Likewise, bimodal peaks centered at the diploid chinook female x coho male hybrid number (2N = 64) and triploid number (3N = 98) also indicate the presence of diploid and triploid individuals (Figure 22). The absence of such bimodal peaks from heat-shocked coho pure matings and coho female x chinook male crosses indicated no triploid induction occurred in coho maternal side crosses (Figures 23 and 24). The absence of bimodal distributions from all other non-heated

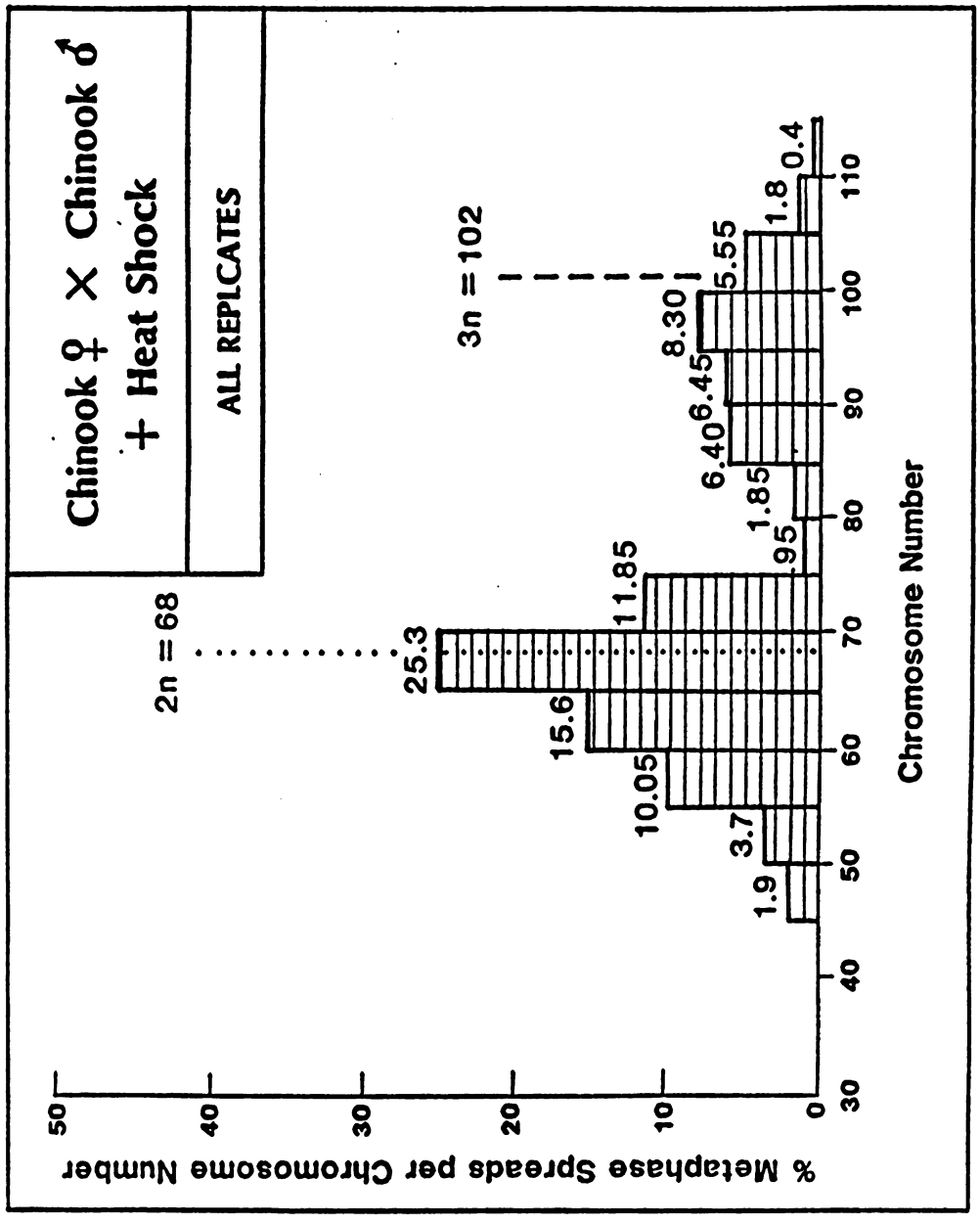


Figure 21. Chromosome number composition of metaphase spreads sampled from heat shocked chinook salmon.

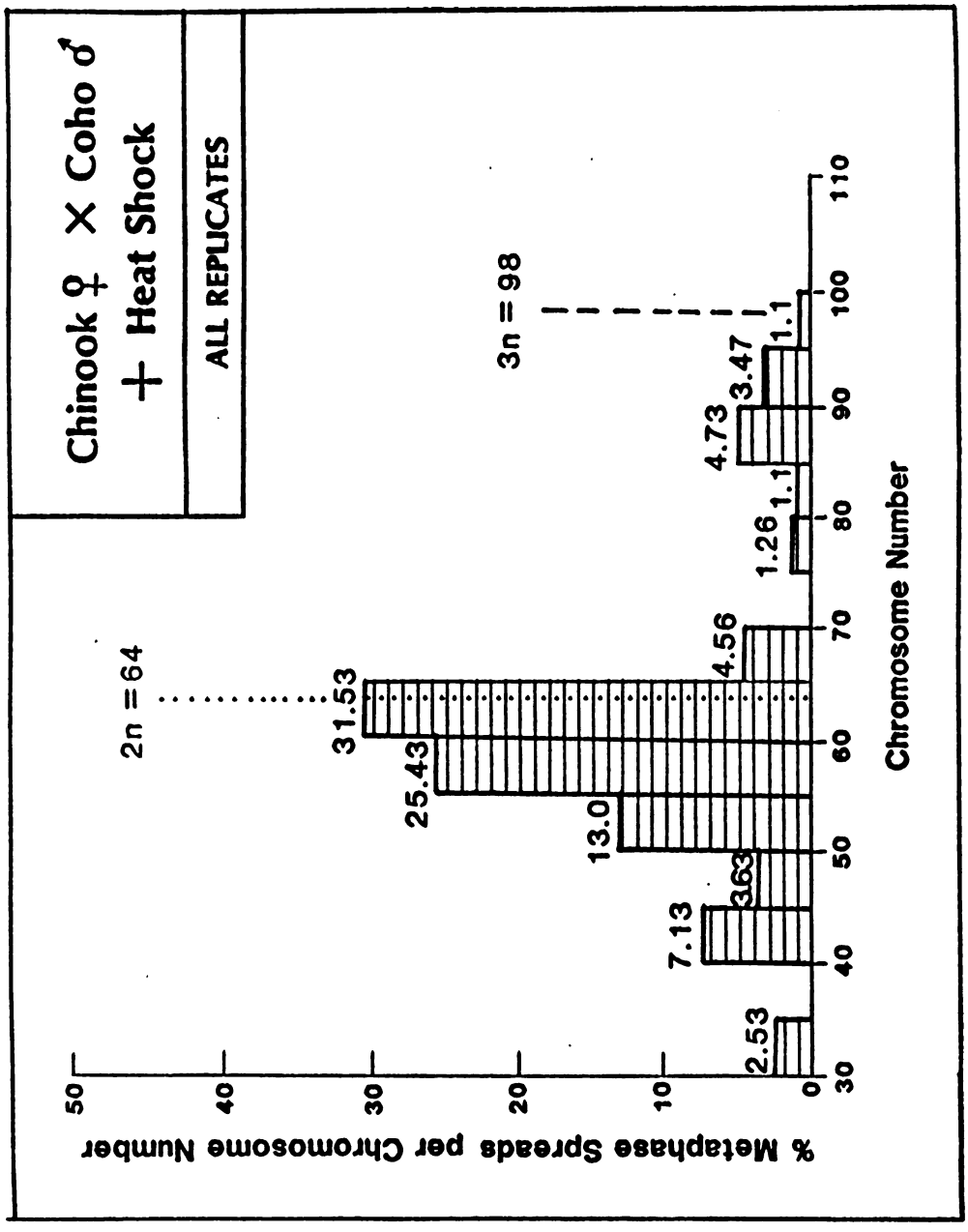


Figure 22. Chromosome number composition of metaphase spreads sampled from heat shocked chinook female x coho male hybrid salmon.

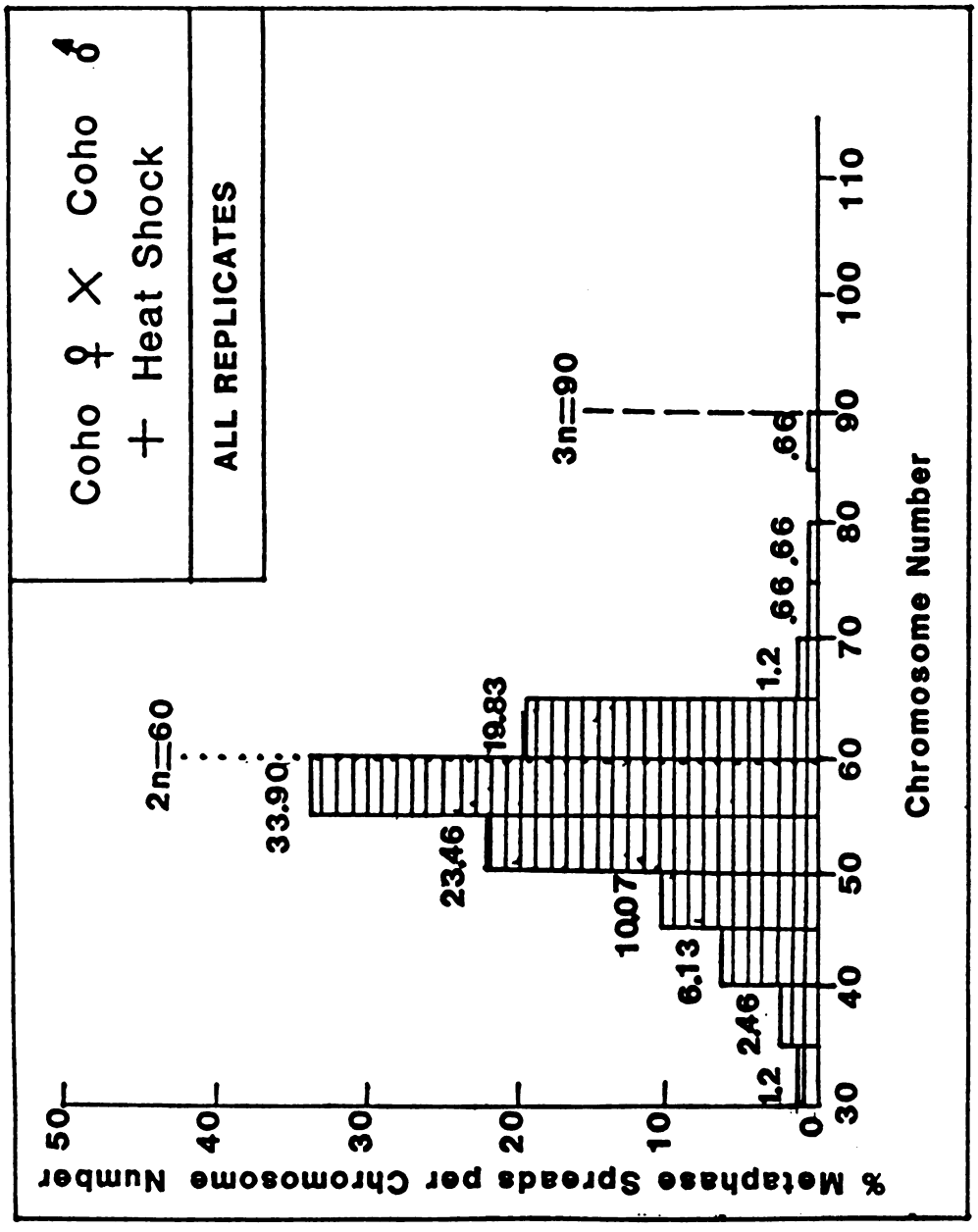


Figure 23. Chromosome number composition of metaphase spreads sampled from heat shocked coho salmon.

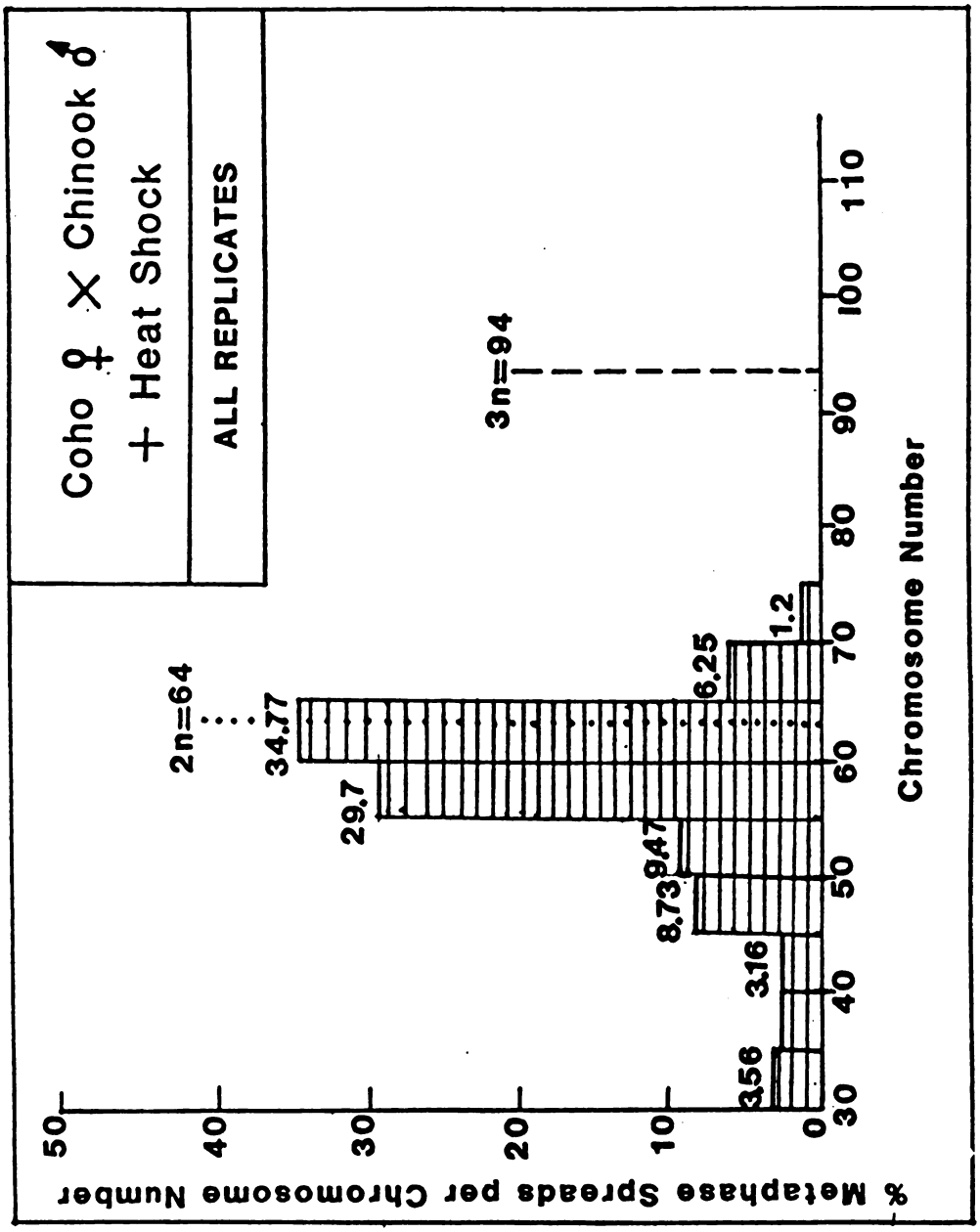


Figure 24. Chromsome number composition of metaphase spreads sampled from heat shocked coho female x chinook male hybrid salmon.

shocked crosses indicated that no natural or hybrid produced triploid individuals were encountered (Figures 8, 11, 14, and 15). It is important to determine that no natural triploids are in the population because natural triploids have been found in rainbow trout (Thorgaard and Gall, 1979) and pink salmon (Utter et al., 1983).

Further evidence of triploid induction comes from examination of nearly complete karyograms of triploid chinook salmon. A triploid chinook salmon would be expected to have one diploid (2N) plus one haploid (1N) set of chromosomes. Using this guideline, plus the standard karyological composition of chinook salmon, a triploid chinook salmon would be expected to have a total of 51 to 54 metacentric-submetacentric chromosomes from 34 to 36 diploid plus 17 to 18 haploid metacentric-submetacentric chromosomes. Likewise, 48 to 51 acrocentric-telocentric chromosomes would be expected in a triploid from a diploid count of 32 to 34 plus a haploid number of 16 to 17 acrocentric-telocentric chromosomes. When analyzed, the two nearly complete triploid karyograms (Figures 25 and 26) come very close to the expected counts. The karyogram represented in Figure 25 contained 96 chromosomes (expected 3N = 102) with 50 metacentric-submetacentric (expected 51 to 54) and 46 acrocentric-telocentric chromosomes (expected 48 to 51). The karyogram in Figure 26 is more similar to the expected counts with 101 chromosomes (expected 3N = 102). Fifty of these chromosomes were metacentric-submetacentric (expected 51 to 43) and 51 acrocentric-telocentric (expected

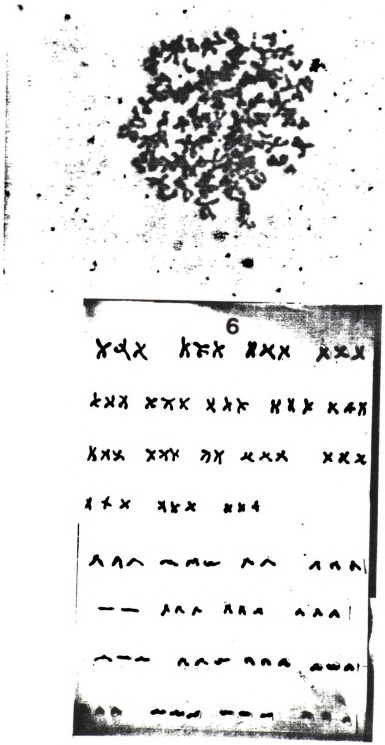


Figure 25. Metaphase spread (above) and karyogram (below) of chinook salmon triploid with 96 chromosomes (50 metacentric-submetacentrics and 46 acrocentric-telocentrics).

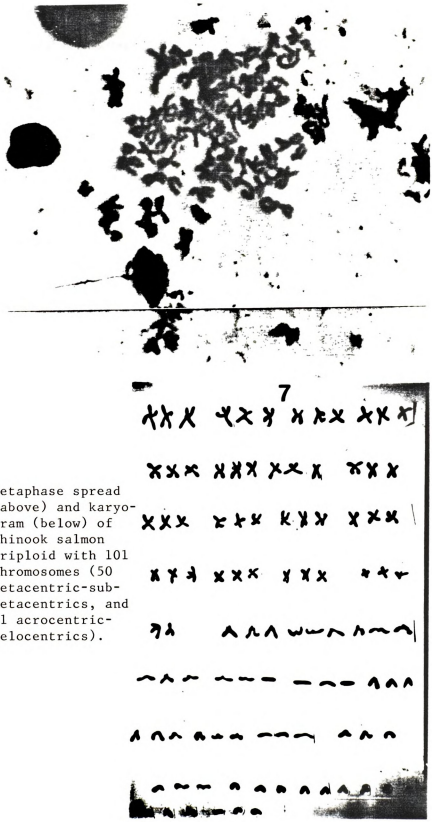


Figure 26. Metaphase spread (above) and karyogram (below) of chinook salmon triploid with 101 chromosomes (50 metacentric-submetacentrics, and 51 acrocentric-telocentrics).

48 to 51). In addition to the total chromosome counts indicating triploidy, the natural grouping of homologous trios of chromosomes seems to occur (Figures 25 and 26). The chance of two or more partial diploid metaphase spreads combining to produce a pseudo-triploid metaphase spread seems remote due to the closeness of the expected counts with the actual counts and the homologous trios evident in Figures 25 and 26.

Comparisons of Efficiency of Various Triploidy Induction Techniques

Although triploidy was induced by heat shock of 36°C for one minute 10 minutes post fertilization in chinook salmon and chinook female x coho male salmon hybrids, the percentage of triploids in each test group is not high enough for production. Triploidy was induced in 42.1 $\pm$ 11% and 21.1 $\pm$ 9% of the individuals in replicate one and two of heat-shocked chinook salmon (Table 1). Heat shock also induced triploidy in 0%, 10 $\pm$ 9.5%, and 14.3 $\pm$ 11% of the individuals in replicates one, two and three of heat shocked chinook female x coho male salmon hybrids (Table 1). This heat shock apparently was not effective at inducing triploidy in coho salmon and coho female x chinook male salmon hybrids since none were observed (Table 1).

Close to 100% triploid induction is desirable because triploid individuals can only be detected from diploids by extensive karyological or cytological techniques. This process would be uneconomical for large hatchery operations. Using the same

Table 1. Percent triploid individuals per treatment group.

Replicate Number	Cross and Treatment Maternal x Paternal	Percent Triploid ± Standard Error
1	Coho x Coho	0
2	Coho x Coho	0
3	Coho x Coho	0
1	Coho x Coho + Heat Shock	0
2	Coho x Coho + Heat Shock	0
3	Coho x Coho + Heat Shock	0
1	Coho x Chinook	0
2	Coho x Chinook	0
1	Coho x Chinook + Heat Shock	0
2	Coho x Chinook + Heat Shock	0
3	Coho x Chinook + Heat Shock	0
1	Chinook x Chinook	0
2	Chinook x Chinook	0
3	Chinook x Chinook	0
1	Chinook x Chinook + Heat Shock	42.1±11
2	Chinook x Chinook + Heat Shock	21.1± 9
1	Chinook x Coho	0
2	Chinook x Coho	0
3	Chinook x Coho	0
1	Chinook x Coho + Heat Shock	10.0±9.5
2	Chinook x Coho + Heat Shock	0
3	Chinook x Coho + Heat Shock	14.3±11

heat shock as in this study, Thorgaard et al., (1981) produced an average of 40% triploid rainbow trout with 30% survival twenty days post fertilization. When compared to this study's average of 18% triploid induction with survival of heat shocked groups ranging from 45-60% at swim up, a large improvement in triploid induction rate with some sacrificing of survival can be accepted. The lower success rate of the chinook salmon and chinook female x coho male salmon hybrids triploid induction compared to rainbow trout triploid induction rate may possibly be attributed to larger egg size of the salmon. Larger eggs may require high temperatures, longer heating times, or a more effective heating technique to be effective. Ways of improving the triploid induction rate must concentrate on these factors.

Improvement of Triploidy Induction Techniques (Heat Shocking)

One way to improve triploid induction rate would be to create a flow-through heat shocking apparatus rather than statically heat shocking the eggs. When a cluster of eggs are heat shocked, eggs located toward the center of the egg mass may not be heated as thoroughly. These eggs may not be successfully triploid induced. A flow through system, in which heated water is pumped through the egg mass, would help in this situation by moving warm water to the center of the egg mass. This would lead to more even and thorough heat shocking with presumably higher triploid induction rates.

Other ways to improve triploid induction rate may be to increase heat-shocking temperatures or duration. Both duration and temperature probably should not be increased dramatically together or excessive mortalities may result. One method may be to lower heat-shock temperatures somewhat (temperatures in high 20°C range instead of 30°C range) while increasing the duration of the shock. For example, rainbow trout have had triploid-induction rates of 50-100% (depending on the strain of trout) with heat shocks of 27°C to 28°C, for 10-15 minutes, at 40 minutes post fertilization. Survival for the heat shocked groups was 30% at hatching compared to the control at 59% (Lincoln and Scott, 1983). Utter et al., (1983) also induced high percentages of triploids (58-84%) in Pacific salmon. This was accomplished with a 28-30°C heat shock, for 10 minutes, ten minutes post fertilization. Several authors induced 100% triploidy in various salmonids. A 26°C heat shock, for 10 minutes, 1 minute post fertilization produced 100% triploid rainbow trout (Solar et al., 1984). Heat shocking of Atlantic salmon eggs resulted in 100% triploids in two different cases. The first case used a heat shock of 32°C, for 5 minute duration within 20 minutes post fertilization (Benfey and Sutterlin, 1984b); and the second case a heat shock of 30°C, for 10-14 minute duration, at 10 to 20 minutes post fertilization (Johnstone, 1985). Johnstone (1985) prefers to use triploid yield (triploid induction rate x percent survival at hatch) as a successful triploid induction scale. Using this technique, Johnstone reports that 100% triploid

induction rates may not be optimal because of lower survival rates that decrease yield. He states that a heat shock of 26°C, for 20 minutes, starting immediately after fertilization produces 94% (instead of 100%) triploid individuals but with highest possible yield (70.5) of triploid individuals because of higher survival. However, if losses due to egg mortalities may be compensated for by slightly larger egg takes, a premium can be placed on production of 100% triploid individuals. Recent communications received by Michigan State University from Hill et al., (1985) also supports medium temperature (28.5°C) heat shocks of medium duration (10 to 15 minutes) to induce triploidy in 100% of the chinook salmon shocked. These workers also found that a slow cooling period rather than fast cooling, after the heat shock enhanced the production of triploid chinook. Preliminary work at Michigan State University supports this supposition. Heat shocks at 28.5°C, for 10 or 15 minutes starting ten minutes after fertilization, followed by a 30 minute slow cooling period induced triploidy in 100% chinook salmon (Westerhof, personal communication).

Improvement of Triploidy Induction (Hydrostatic Pressure)

Another method to induce triploidy in salmonids which may actually prove to be better than heat shocking, is the use of hydrostatic pressure. Pressure shocks work on the same principles as heat shocking, in that the second meiotic division is disrupted or prevented during the period immediately post

fertilization. Pressure shocks of 10,000 psi, for eight minutes, starting 60 minutes from oviposition, induced 97% triploid efficiency (percent triploidy x percent survival) in the axolotl salamander (Gillespie and Armstrong, 1979). The great advantage in pressure shocks over heat shock is higher percent survival with comparatively high triploid induction rates. Pressure shocks have successfully induced triploidy in some salmonids. Rainbow trout eggs, subjected to 7,000 psi of pressure for four minutes, 40 minutes post fertilization had 100% triploid induction rates with over 70% survival 50 days after hatching (Chourrout, 1984). Triploidy was also induced in 100% of Atlantic salmon, using 10,150 psi of pressure applied for three to six minutes within 20 minutes of fertilization. These Atlantic salmon had 70-90% survival compared to only 18% survival of identically heat shocked groups (Benfey and Sutterlin, 1984b). Benfey and Sutterlin stressed that pressure shocks above 10,150 psi, or for longer durations than six minutes, or shocks applied after 20 minutes post fertlization caused 100% mortalities. Obviously extreme care must be taken when using pressure shocks. The high incidence of triploidy and high survival of pressure-shocked eggs makes this a viable technique to be attempted on coho and chinook salmon. The primary disadvantage to pressure shocking is the cost associated with acquiring the specialized equipment suitable to the task.

Failure of Triploid Induction in Coho Salmon

Failure of triploid induction in coho maternal side crosses in this trial is difficult to explain. Coho salmon are liable to triploid induction because a heat shock of 28-30°C for 10 minutes, ten minutes post fertilization induced triploidy in 50% of the coho individuals (Utter et al., 1983). Utter et al. (1983) also had some heat shocking trials which failed to produce triploids in coho salmon. These trials were conducted at temperatures of 33, 34 and 35°C for one minute at ten minutes post fertilization which is almost identical to my heat shocking regiment. One explanation arises from the observation that coho were not at peak ripeness, or at the peak of their spawning run when eggs were taken for this experiment. It is possible that this overripe or underripe condition may be responsible for triploid-induction failure. Other authors have stated the susceptibility to triploid induction may be related to the degree of egg ripeness and timing of ovulation (Lou and Purdom, 1984; Thorgaard and Gall, 1979). I hypothesize that eggs taken late in the spawning season may have progressed into stages of the second meiotic division which cannot be prevented by heat shock. Likewise, eggs taken before they are ripe may not have progressed into a stage which makes them liable to heat shock. This hypothesis may explain why coho salmon, which were not at peak ripeness, were not successfully triploid induced. This hypothesis is further supported by observations on heat-shocking trials performed on chinook salmon, by Michigan State University,

in the fall of 1985. In these trials, a heat-shocking technique which produced 100% triploid individuals in the middle of the spawning run, produced no triploids at the very end of the spawning run. The only other difference in technique between these two groups was the immediate fertilization of eggs after oviposition in the early spawning run and delayed fertilization of eggs in the late season spawning run. The delayed fertilized eggs were stripped and packed dry for a several hour trip to Wolf Lake State Fish Hatchery for subsequent fertilization and heat shocking. It is also possible that eggs transported in this manner prior to fertilization may undergo the second meiotic division rendering a heat shock useless. It is doubtful that this is true because Utter et al., (1983) and Hill et al., (1985) had successful triploid induction using delayed fertilization. If this were true, the question of "What stimuli induces the second meiotic division?" would have to be answered. Could the second meiotic division be stimulated by time after oviposition, physical shock from eggs being transported, or is it fertilization? Another question which needs to be answered is, "What effect does early, mid, or late season egg taking have on triploid induction rates?" If there are correlated differences in triploid induction rate with egg ripeness the reason why these differences exist and what relation does this have to the formation and function of the spindle apparatus of the second meiotic division must be answered before optimal triploid production can occur.

Solid Tissue Karyotyping

Not only are improvements needed in triploid-induction techniques, but triploid-detecting methods have many flaws and need some improvement. Solid-tissue karyotyping, the primary method of ploidy determination used in this study, proved to be very time consuming and labor intensive. Appendix B lists the steps involved with solid-tissue karyotyping and reviews the stages which are critical to the procedure. Karyotyping of teleost fishes in general is more difficult than other vertebrates due to extremely small chromosome size and frequently high chromosome numbers (Blaxhall, 1983a). In addition to this, much time is required in perfecting karyotyping techniques for each species so that optimal colchicine concentrations and exposure times must be determined. If colchicine concentrations are too high or exposure is too long, chromosome morphology is destroyed by condensation (Denton, 1973; Roberts, 1968). The longest (and most identifiable) chromosomes are obtained by the shortest colchicine treatments (Van Tuinen and Valentine, 1982). On the other hand, if inadequate quantities of colchicine, or inadequate periods of colchicine exposure are used, metaphase spreads are rarely obtained. In order to obtain sufficient metaphase spreads for this study, a colchicine exposure time of six to eight hours was needed. This length of treatment, however, caused considerable chromosome condensation resulting in poor chromosome morphology. Identification and classification of metacentric-submetacentric and acrocentric-telocentric chromosomes was difficult because of

condensation. This probably accounts for the differences seen in chromosome classification between, for example, Figures 9 and 10. Both the metaphase spreads in Figures 9 and 10 are of diploid coho salmon with 60 chromosomes. However, Figure 9, which contains chromosomes more condensed than Figure 10, has 44 metacentric-submetacentric chromosomes with 16 acrocentric-telocentric chromosomes. Figure 10, in contrast, has 46 metacentric-submetacentric with 14 acrocentric-telocentric chromosomes. Another good example of the differences encountered in karyograms due to chromosome condensation can be seen in the comparison of chromosome length and detail between Figures 16 and 17. The differences in chromosome condensation probably accounts for the majority of the variation of chromosome classification observed.

Another crucial factor in obtaining an adequate quantity of metaphase spreads is the stage of development and growth of which the individuals are sampled. Since actively dividing cells are required to obtain metaphase spreads, fish in an active stage of growth are required. Salmonids at the blastodisc stage are ideal for good karyograms because chromosomes are larger at this time and cell divisions are frequent (Roberts, 1967). In contrast, eyed embryos of salmonids are undesirable for good karyogram results (Roberts, 1967; Thorgaard, et al., 1982). Similar results were obtained in this study with fingerling salmon. Earlier samples of fingerling salmon which measured approximately six centimeters or less provided more and better quality metaphase spreads than salmon sampled later which measured more than

six centimeters. This was the main reason sample size was reduced from twenty to ten fish per replicate later in the study. Considerably more time was required to process the larger fish because fewer metaphase spreads could be found per preparation and more slide preparations were needed for an adequate number of metaphase spreads to be counted.

Highly variable chromosome counts were another problem associated with solid-tissue karyotyping. This variation is easily seen in the bar graphs in Figures 8, 11, 14, 15, 21, 22, 23 and 24. Chromosome number usually peaks at the expected diploid or triploid chromosome number. The majority of the other counts fall below the expected number and a few usually fall above the expected counts. These variations in counts may be attributed to a variety of causes. First, the high predominance of metaphase spreads containing fewer than the expected chromosome count may be explained by loss of chromosomes during fixation and processing. Chromosomes lost in this manner may not have been fixed adequately to the slide and washed off in later stages of staining and mounting. In addition, in some slides, which were kept in hypotonic KCl solution for slightly longer periods than 30 minutes, or when tissues were not adequately fixed, seemed to contain degraded, fuzzy appearing chromosomes. In these slides it is possible some chromosomes may have been completely degraded and lost. Some chromosomes may also have been hidden from view beneath other chromosomes and cell debris.

The occasional higher than expected chromosome counts must also be explained. Counting errors due to "false centromeres" may explain some of these counts. The procedure of counting chromosomes used the centromere as an indicator of chromosome position within the grid system as explained in Materials and Methods Section. When a metaphase spread was compact, or the chromosomes close together, many chromosome arms overlapped. These overlaps looked like centromeres especially when two darkly stained chromosome arms were involved. These "false centromeres" could have been counted as additional chromosome centromers.

Another possible explanation for higher than expected chromosome counts is that some chromosomes from adjacent or nearby metaphase spreads may have been dislodged from one spread and fixed within another metaphase spread. I do not consider this explanation as very viable because evidence does not support it. Every metaphase spread seemed to be fixed at a different degree of contraction with a characteristic stain color. If chromosomes from different cells mixed, differently stained chromosomes would have been present in one metaphase spread. Only one occurrence of differently stained chromosomes contained within one metaphase spread was observed in my study. Roberts (1967) also observed variations of chromosome number in Atlantic salmon karyotypes. Chromosome counts may also deviate from expected values due to natural causes rather than methodological causes.

Naturally occurring intraspecific variation in chromosome number has been reported in many species. Grammeltredt (1974) and

Fukuoka (1972) found diploid chromosome counts ranging from 58 to 65 in rainbow trout. Different populations of green sunfish (Lepomis cyanellus) show chromosome polymorphism with some populations containing 46 chromosomes and other populations with 48 chromosomes (Becak et al., 1966). Robertsonian translocations, fissions and fusions have been implicated as the cause of these variations in green sunfish and rainbow trout (Becak et al., 1966; Thorgaard, 1976). Thorgaard (1976) also states that many other salmonids, including the coho and sockeye salmon, have been shown to contain Robertsonian polymorphisms. The possibility that some of the chromosome variation found in this study may be do to polymorphisms within coho salmon, chinook salmon, and their hybrids can not be overlooked.

Other problems with solid-tissue karyotyping related to the multiple steps required for the process. If even minor deviations were made in the sequence of steps outlined in Appendix B the quality of metaphase spreads was greatly affected. Notes of the most critical steps used in this technique are also listed in Appendix B. The two most important of these steps appeared to be the use of fresh fixative, and the proper application of the cell suspension to the slide. The use of old fixative was probably the major factor leading to failure of initial karyotyping attempts. Cell suspensions made from inadequately fixed tissues produced very "fuzzy" appearing images of cell particles. In contrast, properly fixed tissues carefully applied to slides produced distinct and sharp images of cell particles and the best

metaphase spreads. Application of the cell suspension to the slide was also critical. Kligerman and Bloom (1977) state, "expel" the cell suspension onto a clean slide. I interpreted this as spreading the cell suspension with some force since most karyotyping techniques require "dropping" cell suspensions onto a slide from various heights (Gold, 1974; Hollenbeck and Chrisman, 1981). In fact, catfish karyotyping was completed by dropping the cell suspension onto slides from a height of three meters (Hollenbeck and Chrisman, 1981). Actually, no force is required in this process and better terminology would be to "flow" the cell suspension onto a hot slide. When cell suspensions were applied with force during the Kligerman and Bloom (1977) procedure, very few cell particles and no chromosome spreads were fixed to the slides.

Lymphocyte and Tissue Culture Karyotyping Techniques

Other karyotyping techniques may give better results than the solid tissue method. Very high quality metaphase spreads with chromosomes of similar size and parallel chromatids are frequently obtained with lymphocyte culturing techniques. A mitotic stimulant, phytohemagglutinin (PHA) used to synchronize cell division followed by a colchicine treatment to stop cells at the same time in metaphase has been used (Wolters et al., 1981a). Tissue culture, using trypsin to emulsify tissue into individual cells, may also achieve good metaphase spreads when treated with PHA and colchicine (Blaxhall, 1975). The main disadvantage with

these two techniques is they require more sophisticated equipment than a microscope, slides, and fixative such as culture media, centrifuges, incubating containers, and incubators. Another disadvantage is that culturing techniques require considerably large amounts of tissue or blood making work on small specimens impossible (Blaxhall, 1981; Denton, 1973). Finally, even though karyotypes may be of better quality with cell culturing techniques, results may still vary depending on the individual person's technique (Grammeltvedt, 1975; Hartley and Horn, 1983).

The main advantages of solid-tissue karyotyping is that it does not require highly specialized equipment or training and is relatively inexpensive (Thorgaard, et al., 1982). Karyotyping is also a direct way of determining triploidy (by counting chromosomes) and it is the only consistent way to confirm if hybridization or gynogenesis has occurred in experimental hybrid crosses. The main disadvantages of solid-tissue karyotyping are that it is labor intensive and time consuming, resulting in relatively small sample sizes to determine triploid induction rates. Due to the small sample size, large standard errors are associated with the percentage of triploid individuals induced. For example, in Table 1 in the first replicate of chinook females x coho males + heat shock, the percentage of triploid individuals is 10% $\pm$ 9.5%. In order to reduce this large standard error, larger samples must be taken. If larger samples are needed, a better triploid identification technique is required.

Erythrocyte Parameters Used for Triploid Identification

All other triploid-identification methods indirectly measure the amount of DNA contained within the cell nuclei. Erythrocyte parameters can be used for triploid identification. This system works on the premise that triploids, having an additional haploid (1N) set of chromosomes with more DNA, will have larger nuclei and cells than diploids. In fishes nuclear measurements are usually performed on erythrocytes since fish erythrocytes are nucleated and are readily available by venous puncture. The simplest and most inexpensive method of determining triploidy is measuring parameters of erythrocytes and erythrocyte nuclei. Erythrocyte parameters have previously been used to identify triploids and diploids of hybrid grass x bighead carp (Beck and Biggers, 1983), Atlantic salmon (Benfey et al., 1984), Poeciliopsis (Cimino, 1973), and channel catfish (Wolters, et al., 1982c). In this study a comparison of diploid and triploid erythrocyte distributions in relation to various nuclei parameters reveals a difference in nuclei size between diploid and triploid chinook salmon (see the bar graphs of Figures 27-30). The means, standard errors, and triploid/diploid ratios of the various parameters are listed in Table 2. Ideally, the ratio of triploid nuclei volume to diploid nuclei volume should be 1.5:1. In this study, the actual ratio observed was 1.96:1 and probably indicates that the nuclei are probably not true ellipses so the formula overestimates the volume. Based on the means and the sizes of the standard error, the best indicator of triploidy was

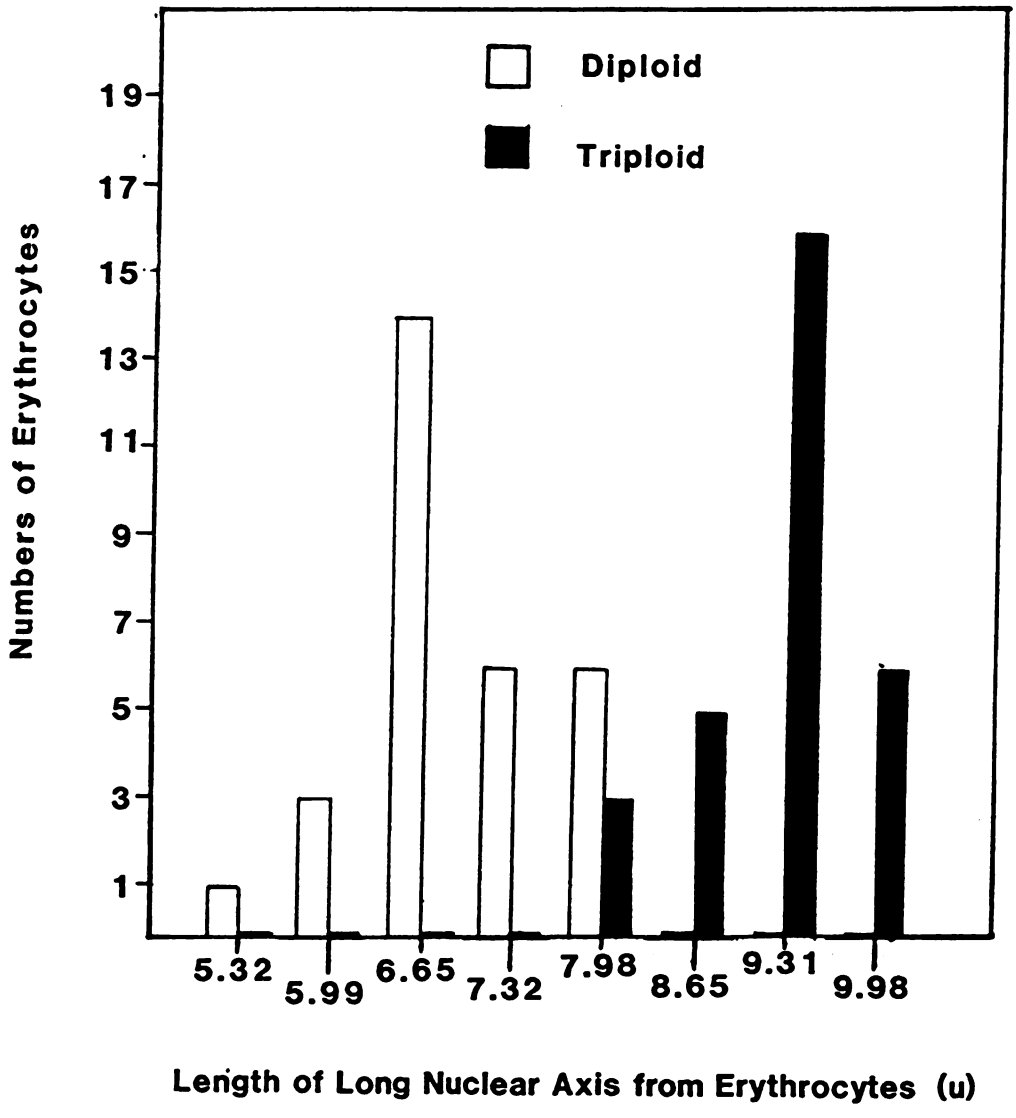


Figure 27. Distribution of diploid and triploid erythrocytes per length of long nuclear axis.

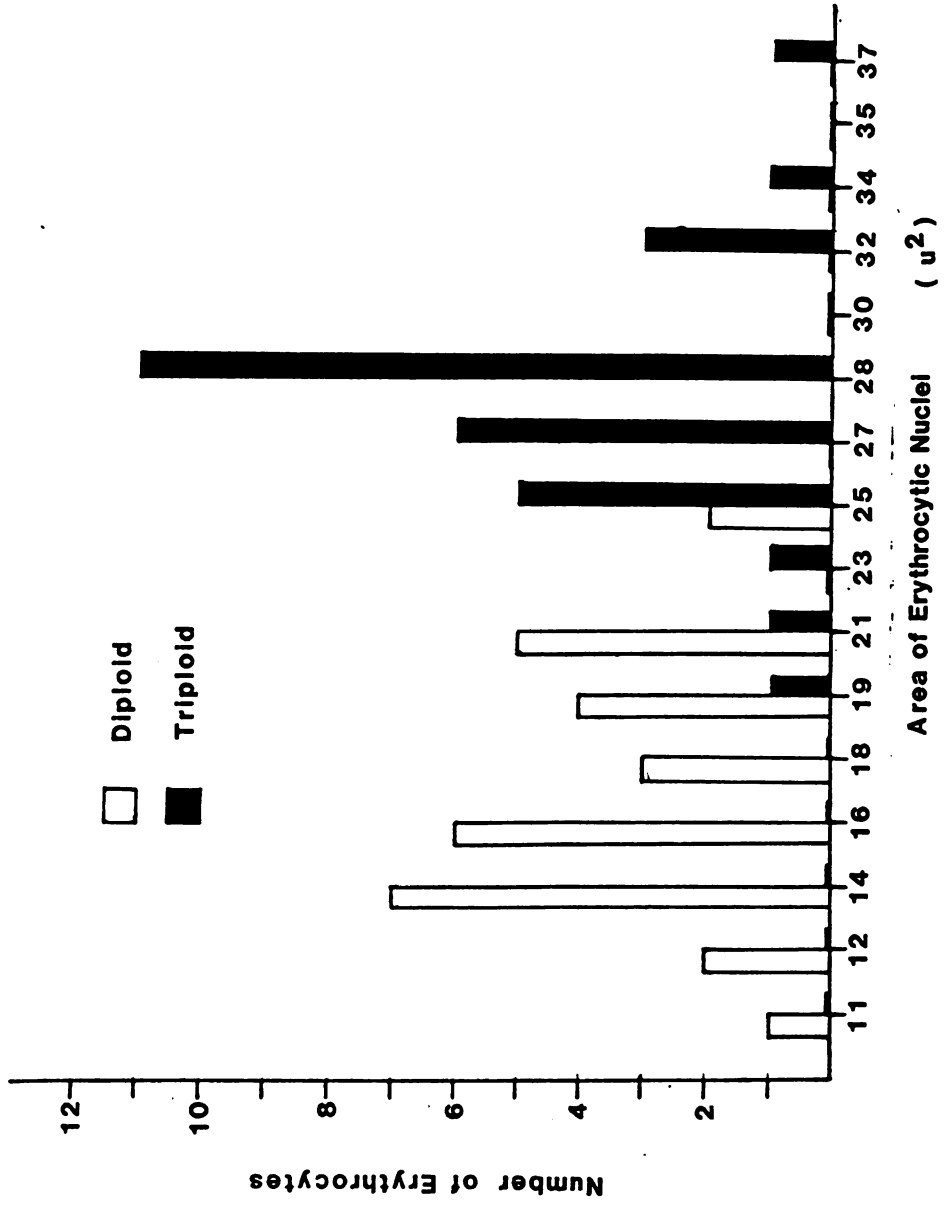


Figure 28. Distribution of diploid and triploid erythrocytes per area of erythrocytic nuclei.

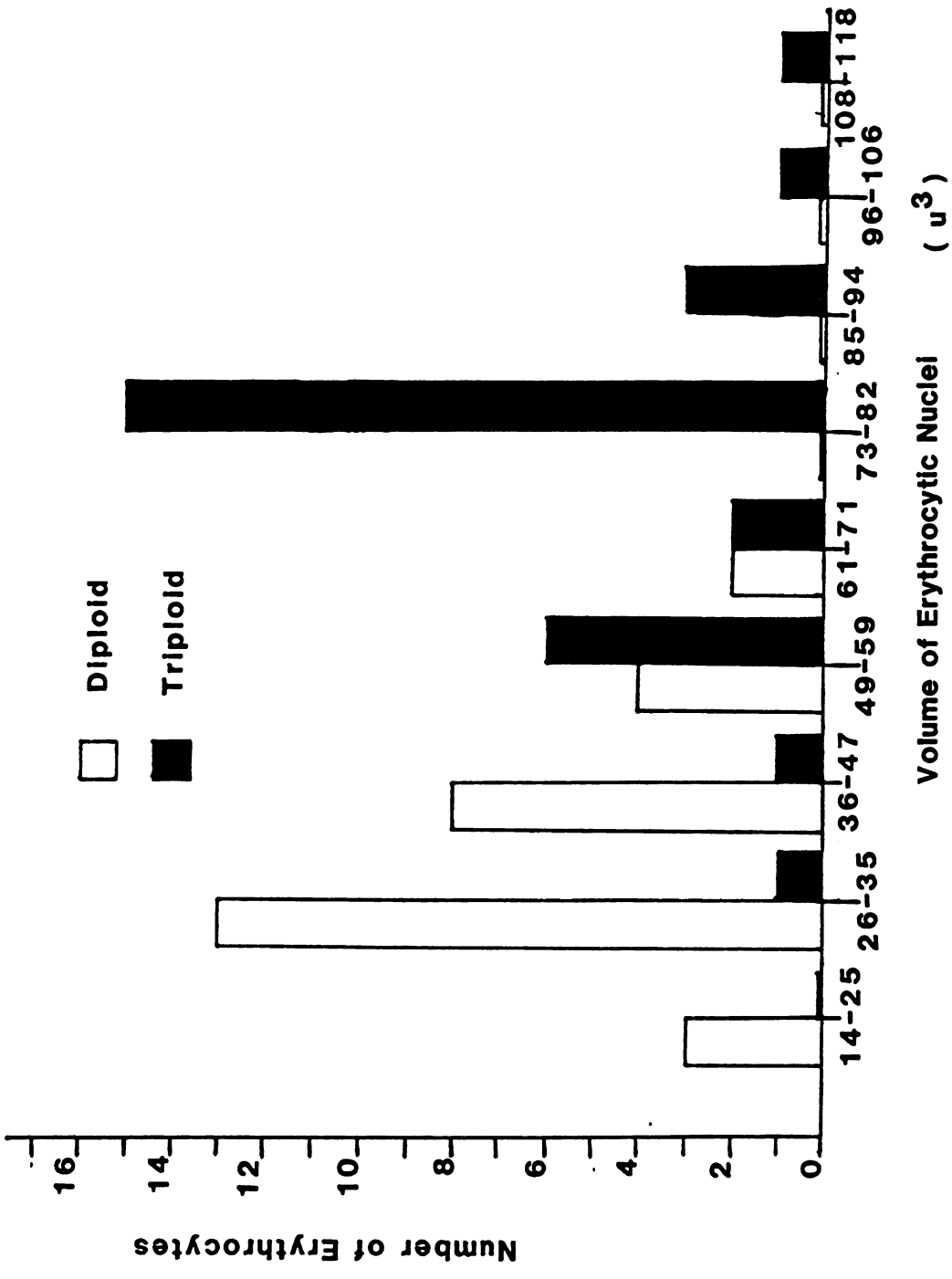


Figure 29. Distribution of diploid and triploid erythrocytes per volume of erythrocytic nuclei.

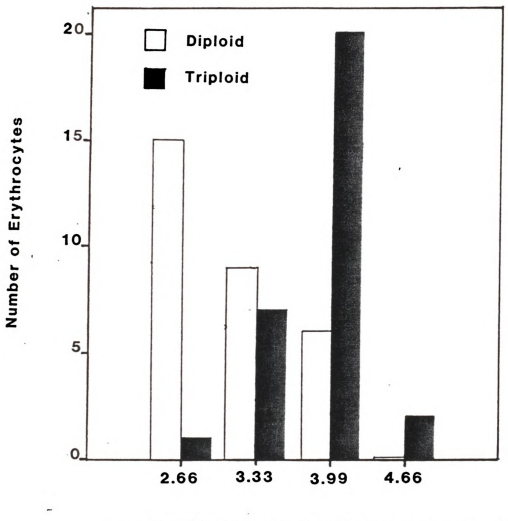


Figure 30. Distribution of diploid and triploid erythrocytes per length of short nuclear axis.

Table 2. Nuclear parameters of erythrocytes from triploid and diploid chinook salmon.

	Long Axis Length (u)	Short Axis Length (u)	Area (u^2)	Volume (u^3)
Triploid (3N)	9.20±.58	3.83±.42	27.73±3.60	71.77±16.13
Diploid (2N)	6.94±.69	3.13±.53	17.09±3.58	36.70±13.76
Ratio $\frac{3N}{2N}$	1.33	1.22	1.62	1.96

length of nuclear long axis. That is to say, the smallest amount of overlap between diploid and triploid measurements occurred in length of the long nuclear axis from the erythrocytes. Ranked in increasing amount of overlap and decreasing ability to differentiate triploidy is area of erythrocytic nuclei, followed by volume of erythrocytic nuclei, and finally length of short nuclear axis. These results are very similar to the findings of Wolters et al. (1982c). Wolters et al. (1982c) states that the best method for determining triploidy in channel catfish was to consider all nuclear variables together. Measuring the length of nuclear major axis was the second best method for determining triploidy. Finally, volume of erythrocytic nuclei and length of nuclear minor axis were the least effective means for determining triploidy due to high percentages of improper classifications. Wolters et al. (1982c) stated that although weighted mean values and confidence intervals of erythrocyte measurements may be distinct, the range of measurements still overlap and result in some mis-classification. The reason for the poor discriminating powers of the minor axis is a change in shape of erythrocytes when diploids and triploids are compared. The major axis of the triploid nuclei is increased at a greater rate than the minor axis. Wolters et al. (1982c) also suggested that care must be used when erythrocyte parameters are used to determine triploidy because of the misclassification involved. They suggested that only length of nuclear major axis needs to be used as a discriminating variable for channel catfish triploids.

Erythrocyte nuclear and cellular length was shown to correctly identify triploid and diploid grass carp x bighead carp hybrids with 98% accuracy (Beck and Biggers, 1983). Studies of haematology of triploid landlocked Atlantic salmon also indicated that triploids have increased erythrocyte nuclear and cellular size (Benfey et al. 1984). Five of six erythrocyte parameters, including cell major axis, cell surface area, nuclear major axis, nuclear minor axis and nucleus volume could be used to successfully classify 100% of the individuals as triploids or diploids. Measurement of erythrocyte minor axis was the only parameter which could not determine triploidy with 100% accuracy. Even this measurement was at least capable of 95% accuracy (Benfey et al. 1984). Due to these results, I feel that triploidy may be accurately detected in chinook salmon by measurements of the long nuclear axis from erythrocytes. Mean nuclear lengths of eight microns or greater would indicate a triploid.

Other Methods of Triploid Identification

Other methods have also been used to detect triploidy including coulter counter channelyzer, microspectrophotometry, and flow cytometry. The coulter counter channelyzer works on the principle that triploids have larger erythroycytes than diploids, with the machine sorting erythrocytes according to size. Flow cytometry works by detecting laser excited fluorescence of the stained nuclear DNA (Allen, 1983). Flow cytometry offers the advantages of being very accurate and relatively fast so large

samples can be determined. Other advantages are (a), specimens remain alive (only a small quantity of blood or tissue is required), (b), any tissue is usable, and (c), almost any age of a specimen may be analyzed (Allen, 1983; Thorgaard et al. 1982). The disadvantages of flow cytometry are the prohibitively high cost of the equipment and the high degree of technological training required. Flow cytometry and various erythrocyte size determination techniques are more suitable for triploid determination than solid-tissue karyotyping. These techniques are less time consuming allowing more time to be spent analyzing larger numbers of samples thus reducing the associated standard error.

CONCLUSIONS

1. Heat shocks of 36°C for one minute, initiated at ten minutes post fertilization, induced triploidy in chinook salmon and chinook female x coho male salmon hybrids. Although triploidy was induced, this heat-shock treatment was not considered successful since only 0 to 42% triploid individuals were produced. A triploid induction rate of 100% is desired for practical production uses.

2. The heat-shock treatment used failed to induce triploidy in coho salmon and coho female x chinook male salmon hybrids.

3. The effects of degree of ripeness of eggs on triploid production must be determined before optimal production of triploids is achieved.

4. The effects of immediate fertilization versus delayed fertilization on triploid induction rates must be determined before optimal production of triploids is achieved.

5. The use of chinook female x coho male salmon hybrids is not recommended due to high incidence of malformations which occurred in the fry.

6. In contrast, coho female x chinook male salmon hybrids have very good survival rates with low incidence of disembryogenesis. This may be a viable hybrid to use for production.

7. In order to determine the effects of triploidy on chinook salmon x coho salmon reciprocal hybrids, a method of producing near 100% triploids needs to be used to achieve adequate numbers of triploid hybrids.

8. Solid-tissue karyotyping is a slow and inefficient means for triploid determination. Faster, more accurate methods which are capable of larger sample sizes should be used. Such methods might include flow cytometry, coulter counter channelizer and erythrocytic nuclei size determinations.

9. Based on initial experiments, triploid induction rates of 100% can be achieved by heat shocks of approximately 28^{o}C applied for ten minutes, ten minutes post fertilization if eggs are cooled slowly.

APPENDIX A

Appendix A

Heat-shocking procedures used at Little Manistee Weir, October 15, 1984, to induce triploidy in coho salmon, chinook salmon, and coho x chinook salmon reciprocal hybrids.

A. Set up heat shocking container with river water and stabilize temperature of water at 36°C (approximately 4 hours).

B. Prepare containers to receive eggs (clean, dry and insulated from rapid temperature changes).

C. Strip eggs from three coho females into pan.

1. Mix eggs well

2. Split these in half (one half to be fertilized immediately, the other half saved dry for step C-2-b. Eggs not fertilized immediately but kept dry and cool are viable for at least 4 hours) (Piper et. al., 1983).

a) The first half to be fertilized immediately by six coho males (coho males were not very ripe, (producing less milt) so additional males were needed). (Save some coho sperm for later crosses).

1) Split these in half again.

Half to be false heat shocked ten minutes post fertilization (Cross 1).

Half to be heat shocked ten minutes post fertilization (Cross 2).

b) The second half to be fertilized by three chinook males (save some chinook sperm for later crosses).

1) Split these in half again.

Half to be false heat shocked ten minutes post fertilization (Cross 3).

Half to be heat shocked ten minutes post fertilization (Cross 4).

D. Strip three chinook females into pan.

1. Mix eggs well.

2. Split these eggs in half (one half to be fertilized immediately, the other half saved dry for step D-2-b).

a. The first half to be fertilized by three chinook.

1) Split these in half again.

Half to be false heat shocked (Cross 5).

Half to be heat shocked (Cross 6).

b. The second half to be fertilized by six coho males.

1) Split these in half again.

Half to be false heat shocked (Cross 7).

Half to be heat shocked (Cross 8).

Each separate group of eggs was fertilized consecutively at two-minute intervals. This time allotment was adequate to move the various groups in and out of heat shocking and false heat shocking. All eggs were fertilized in the same ten-minute period to minimize time differences between collection of gametes and fertilization for the different crosses.

APPENDIX B

Appendix B

Solid-tissue karyotyping technique for four month old chinook salmon and coho salmon. Modified and adapted from Kligerman & Bloom, (1977) and from Paul Scheerer, (Washington State University, personal communication).

Step 1: Prepare colchicine solution in .85% sterile saline.

Step 2: Inject fish, intermuscular on dorsal surface just anterior to dorsal fin or interperitoneal with 25 ug colchicine/g fish weight in .85% sterile saline solution.

Step 3: Allow injected fish to swim in aerated water for six to eight hours post injection.

Step 4: Sacrifice fish and remove kidneys and spleen. (Other tissue types may also be removed such as gill arches, intestine, etc.)

Step 5: Place tissues in approximately 10 times their volume of hypotonic .4% KCl solution for 20 to 30 minutes.

Step 6: Remove ALL hypotonic KCl solution and exchange with FRESH 3:1 ethanol (or methanol): acetic acid. Allow tissue to fix for 30 minutes, then exchange original alcohol:acetic acid fixative with fresh fixative. Allow tissues to fix for another 30 minutes, then exchange once again with fresh fixative. (Tissues may be stored at 4°C at this time).

Step 7: Remove tissue from alcohol:acetic acid fixative and blot off excess fixative on filter paper.

Step 8: Place tissue in four to six drops 50% acetic acid in depression slide or small porcelain watch glass. Mince tissue gently with bottom of test tube for one minute until completely emulsified.

Step 9: Aspirate suspension in a micro-capillary tube and transfer to a hot glass microscope slide (40-45°C) in a ring of about 1 cm in diameter.

Wait four to five seconds, then draw off excess fluid not dried to slide.

Repeat this process several times to produce several rings at different locations on the slide.

(It is critical not to get too high of a concentration of cells in the suspension. If too many cells are in the suspension then too many cells will dry on the slide and impede spreading of chromosomes and single cell resolution).

Step 10: Stain slides in 4% Giemsa in .01 M phosphate buffer at pH 7; or in 100% Giemsa for six minutes with 1.5 N NH_4OH used as intensifier for two minutes.

Step 11: Rinse slides in tap water or distilled water. Allow slides to air dry.

Step 12: Fix slides for 10 minutes in Xylene.

Step 13: Mount slides in Apochromount, Eukitt, or other suitable mounting media.

Step 14: Scan slides at 100 X along periphery of rings. Spreads will appear as small dots among cells.

Step 15: View spreads at 1,000 X.

Notes of critical procedures found in this technique:

Step 2 - Various authors report fish may be allowed to swim in colchicine solution. This method never produced good results in my study.

Step 5 - Amount and length of treatment of tissue in hypotonic solution seems moderately critical. If tissue is placed in too large a volume of hypotonic solution or for too long a time (> 30 minutes), cells seem to swell excessively, possibly rupturing and losing chromosomes.

Step 6 - Removing ALL hypotonic solution and using FRESH fixative seems crucial to proper fixation of tissues. Fresh alcohol:acetic acid fixation leads to good chromosome preparations later. The use of old fixative was probably the single most importance factor for initial failure of karyotyping. Methanol:acetic acid fixative begins to degrade to methyl acetate within 30 minutes after mixing. Methyl acetate is not an effective fixative (Denton, 1973).

Step 8 - It is important not to mince tissue excessively. Experience may be your only guide here.

Step 9 - Several critical phases are involved in this step. Kligerman and Bloom (1977) state, "expel" the cell suspension onto a clean slide. I interpreted this as spreading

the cell suspension with some force since most karyotyping techniques require "dropping" cell suspensions from various heights (Gold, 1974; Hollenbeck and Chrisman, 1981). Actually, no force is required in this process and better terminology would be to "flow" the cell suspension onto a hot slide. Applying the suspension with any force may impede the formation of good chromosome preparations.

It is also critical not to get too high of a concentration of cells in the suspension. If excessive cells are in the suspension and dry on the slide, then single cell resolution and chromosome spreading may be impeded.

REFERENCES

Allen, Jr., S.K. 1983. Flow cytometry: assaying experimental polyploid fish and shellfish. Aquaculture 33:317-328.

Allen, Jr., S. and J. Stanley. 1979. Polyploid mosaics induced by cytochalasin B in landlocked Atlantic salmon (Salmo salar). Trans. Am. Fish. Soc. 108:462-466.

Allen, Jr., S. and J. Stanley. 1983. Ploidy of hybrid grass carp x bighead carp determined by flow cytometry. Trans. Am. Fish. Soc. 112:431-435.

Al-Sabti, K. 1985. Chromosomal studies by blood leukocyte culture technique on three salmonids from Yugoslavian waters. J. Fish. Biol. 26:5-12.

Battle, H.I. 1944. The embryology of the Atlantic salmon (Salmo salar). Can. J. of Res. 22(5):105-125.

Becak, W., M.L. Becak and S. Ohno. 1966. Intraindividual chromosomal polymorphism in green sunfish (Lepomis cyanellus) as evidence of somatic segregation. Cytogenetics. 5:313-320.

Beck, M.L. and C.J. Biggers. 1983. Erythrocyte measurements of diploid and triploid Ctenopharyngodon idella ♀ x Hypothalmichthys nobilis ♂ hybrids. J. Fish. Biol. 22:497-502.

Behler, J.L. and F.W. King. 1979. The Audubon Society Field Guide to North American Reptiles and Amphibians. Chanticleer Press, Inc., New York. 719 pp.

Benfey, T.J. and A.M. Sutterlin. 1984a. Growth and gonadal development in triploid landlocked Atlantic salmon (Salmo salar). Can. J. Fish. Aquat. Sci. 41:1387-1392.

Benfey, T.J. and A.M. Sutterlin. 1984c. The haematology of triploid Atlantic salmon (Salmo salar). J. Fish. Biol. 24:333-338.

Benfey, T.J., A.M. Sutterlin and R.J. Thompson. 1984. Use of erythrocyte measurements to identify triploid salmonids. Can. J. Fish. Aquat. Sci. 41:980-984.

Beverton, R.J.H. and S.J. Holt. 1957. On the dynamics of exploited fish populations. U.K. Min. Agric. Fish., Fish. Invest. (Ser. 2). 19:533.

Blanc, J. and B. Chevassus. 1979. Interspecific hybridization of salmonid fish I. Hatching and survival up to the 15th day after hatching in F-1 generation hybrids. Aquaculture. 18:21-34.

Blanc, J. and B. Chevassus. 1982. Interspecific hybridization of salmonid fish II. Survival and growth up to the 4th month after hatching in F-1 generation hybrids. Aquaculture. 9:383-387.

Blanc, J.M. and H. Poisson. 1983. Parental sources of variation and hatching and early survival rates of Salmo trutta x Salvelinus fontinalis hybrid. Aquaculture. 32:115-122.

Blaxhall, P.C. 1975. Fish chromosome techniques a review of selected literature. J. Fish. Biol. 7:315-320.

Blaxhall, P.C. 1981. A comparison of methods used for the separation of fish lymphocytes. J. Fish. Biol. 18:177-181.

Blaxhall, P.C. 1983a. Chromosome karyotyping of fish using conventional and G-banding methods. J. Fish. Biol. 22:417-424.

Blaxhall, P.C. 1983b. Factors affecting lymphocyte culture for chromosome studies. J. Fish. Biol. 22:61-76.

Briggs, R. 1947. The experimental production and development of triploid frog embryos. J. Exp. Zool. 106:237-265.

Buss, K. and J.E. Wright. 1956. Results of species hybridization within family Salmonidae. Prog. Fish. Cult. October, 1956, p. 149-156.

Cassani, J.R., W.E. Caton and B. Clark. 1984. Morphological comparisons of diploid and triploid hybrid grass carp Ctenopharyngodon idella ♀ x Hypothalmichthys nobilis ♂. J. Fish. Biol. 25:269-278.

Chevassus, B. 1979. Hybridization in salmonids - results and perspectives. Aquaculture. 17:113-128.

Chevassus, B. 1983. Hybridization in fish. Aquaculture. 33:245-262.

Chourrout, D. 1980. Thermal induction of diploid gynogenesis and triploidy in the eggs of the rainbow trout (Salmo gairdneri Richardson). Reprod. Nutr. Develop. 20:727-733.

Chourrout, D. 1984. Pressure induced retention of second polar body and suppression of first cleavage in rainbow trout: production of all triploids, all tetraploids, and heterozygous and homozygous diploid gynogenetics. Aquaculture. 36:111-126.

Chourrout, D. and J. Itskovich. 1982. Three manipulations permitted by artificial insemination in Tilapia: induced diploid gynogenesis, production of all triploid populations and intergeneric hybridization. International Symposium on Tilapia Proceedings, Nazareth, Israel, May 8-13.

Chourrout, D. and E. Quillet. 1982. Induced gynogenesis in the rainbow trout: Sex and survival of the pregenies production of all triploid populations. Theor. Appl. Genet. 63:201-205.

Cimino, M.C. 1973. Karyotypes and erythrocyte sizes of some diploid and triploid fishes of the genus Poeciliopsis. J. Fish. Res. Bd. Can. 30:1736-1737.

Denton, T.E. 1973. Fish Chromosome Methodology. C.C. Thomas Publ., Illinois.

Dunham, R.A., R.O. Smitherman, M.J. Brooks, M. Benchakan and J.A. Chappell. 1982. Paternal predominance in reciprocal channel-blue hybrid catfish. Aquaculture. 29:389-396.

Elinson, R.P. and A. Briedis. 1981. Triploidy permits survival of an inviable amphibian hybrid. Developmental Genetics. 2:357-367.

Ferrier, V. and A. Jaylet. 1978. Induction of triploidy in the newt Pleurodeles waltlii by heat shock or hydrostatic pressure. Chromosoma. 69:47-63.

Frankhauser, G. and R.R. Humphrey. 1950. Chromosome number and development of progeny of triploid axolotl females mated with diploid males. J. Exper. Zool. 115(2):207-241.

Fukuoka, H. 1972. Chromosome number variations in the rainbow trout (Salmo gairdneri irideus (Gibbons)). Japan. J. Genetics. 47(6):455-458.

Garling, Jr., D.L. and M. Masterson. 1985. Survival of Lake Michigan chinook salmon eggs and fry incubated at three temperatures. Prog. Fish. Cult. 47(1):63-66.

Gervai, J., S. Peter, A. Nagy, L. Horvarth and V. Csanyi. 1980. Induced triploidy in carp, Cyprinus carpio L. J. Fish. Biol. 17:667-671.

Gillespie, L.L. and J.B. Armstrong. 1979. Induction of triploid and gynogenetic diploid axolotls (Ambystoma mexicanum) by hydrostatic pressure. J. Exp. Zool. 210:117-122.

Gill, J.L. 1978. Design and Analysis of Experiments in the Animal and Medical Sciences. Volume I. Iowa State University Press, Ames, Iowa.

Gjedrem, T. 1976. Possibilities of genetic improvements in salmonids. J. Fish. Res. Board. Com. 33:1094-1099.

Goetz, F.W., E.M. Donaldson, G.A. Hunter and H.M. Dye. 1979. Effects of estradiol-17B and 17-α methyltestosterone on gonadal differentiation in the coho salmon (Oncorhynchus kisutch). Aquaculture. 17:267-278.

Gold, J.R. 1974. A fast and easy method for chromosome karyotyping in adult teleosts. Prog. Fish. Cult. 36(3):169-171.

Gold, J.R., W.J. Karel and M.R. Strand. 1980. Chromosome formulae of North American fishes. Prog. Fish. Cult. 42(1):10-23.

Grammeltvedt, A. 1974. A method of obtaining chromosome preparations from rainbow trout (Salmo gairdneri) by leucocyte culture. Norw. J. Zool. 22:129-134.

Grammeltvedt, A. 1975. Chromosomes of salmon (Salmo salar) by leukocyte culture. Aquaculture. 5:205-209.

Hartley, S.E. and M.T. Horne. 1983. A method for obtaining mitotic figures from blood leucocyte cultures of rainbow trout, Salmo gairdneri. J. Fish. Biol. 22:77-82.

Hill, J., A. Hickerson, D. Sheldon, and K. Warren. 1985. Production of triploid chinook salmon (O. tshawytscha) using flow through heat shock and subsequent gradual cooling. Clatsop Economic Development Comm. Fisheries Proj. Astoria, Oregon (unpublished paper).

Hollenbeck, P.J. and C.L. Chrisman. 1981. Kidney preparations for chromosomal analysis of Ictaluridae. Copeia. 1:216-217.

Johnstone, R. 1985. Induction of triploidy in Atlantic salmon by heat shock. Aquaculture. 49:133-139.

Kligerman, A.D. and S.E. Bloom. 1977. Rapid chromosome preparations from solid tissues of fishes. J. Fish. Res. Board. Can. 34:266-269.

Leitritz, E. and R.C. Lewis. 1980. Trout and Salmon Culture (Hatchery Methods). California Fish Bulletin, Number 164.

Lemoine, Jr., H.L. and L.T. Smith. 1980. Polyploidy induced in brook trout by cold shock. Trans. Am. Fish. Soc. 109:626-631.

Levan, A., K. Fredga and A.A. Sandberg. 1965. Nomenclature for centromeric position on chromosomes. Hereditas. 52:201-220.

Lincoln, R.F. 1981. Sexual maturation in triploid male plaice (Pleuronectes platessa) and plaice x flounder (Platichthyes flesus) hybrids. J. Fish. Biol. 19:415-426.

Lincoln, R.F., D. Aulstad, and A. Grammeltvedt. 1974. Attempted triploid induction in Atlantic salmon (Salmo salar) using cold shocks. Aquaculture. 4:287-297.

Lincoln, R.F. and A.P. Scott. 1983. Production of all female triploid rainbow trout. Aquaculture. 30:375-380.

Lincoln, R.F. and A.P. Scott. 1984. Sexual maturation in triploid rainbow trout, Salmo gairdneri Richardson. J. Fish. Biol. 25:385-392.

Loginova, G.A. and S.V. Krasnoperova. 1982. An attempt at crossbreeding Atlantic salmon and pink salmon (preliminary report). Aquaculture. 27:329-337.

Lou, Y.D. and C.E. Purdom. 1984. Polyploidy induced by hydrostatic pressure in rainbow trout, Salmo gairdneri Richardson. J. Fish. Biol. 25:345-351.

McBride, J.R. and A.P. van Overbeeke. 1971. Effects of androgens, estrogens, and cortisol on the skin, stomach, liver, pancreas and kidney in gonadectomized adult sockeye salmon (Oncorhynchus nerka). J. Fish Res. Bd. Can. 28:485-490.

Pillay, T.V.R. (Editor), and W.A. Dill (Editor). 1976. Chapter X genetics and genetic improvement in fish by R. Moaz. Advances in Aquaculture. Fishing News Books Ltd., Farnham, Surrey, England, p. 610-621.

Piper, R.G., I.B. McElwain, L.E. Orme, J.P. McCraren, L.G. Fowler and J.R. Leonard. 1983. Fish Hatchery Management. United States Department of the Interior, Fish and Wildlife Service, Washington, D.C.

Purdom, C.E. 1973. Induced polyploidy in plaice (Pleuronectes platessa) and its hybrid with the flounder (Platichthyes flesus). Heredity. 29:11-24.

Purdom, C.E., D. Thompson and Y.D. Lou. 1985. Genetic engineering in rainbow trout, Salmo gairdnerii Richardson, by the suppression of meiotic and mitotic metaphase. J. Fish. Biol. 27:73-79.

Refstie, T., J. Stoss, E.M. Donaldson. 1982. Production of all female coho salmon (Oncorhynchus kisutch) by diploid gynogenesis using irradiated sperm and cold shock. Aquaculture. 29:67-82.

Refstie, T., V. Vassvik and T. Gjedrem. 1977. Induction of polyploidy in salmonids by cytochalasin B. Aquaculture. 10:65-74.

Roberts, F.L. 1967. Chromosome cytology of the Osteichthyes. Prog. Fish Cult. April, 1967, p. 75-83.

Roberts, F.L. 1968. Chromosome polymorphism in North American landlocked Salmo salar. Can. J. Genet. Cytol. 10:865-875.

Robertson, O.H. 1961. Prolongation of the life span of kokanee salmon (Oncorhynchus nerka kennerlyi) by castration before beginning of gonad development. Zoology. 47:609-621.

Scheerer, P.D. and G.H. Thorgaard. 1983. Increased survival in salmonid hybrids by induced triploidy. Can. J. Fish. Aquat. Sci. 40:2040-2044.

Schreck, C.B. and L.G. Fowler. 1982. Growth and reproductive development in fall chinook salmon: Effects of sex hormones and their antagonists. Aquaculture. 26:253-263.

Schultz, R.J. 1967. Gynogenesis and triploidy in the viviparous fish Poeciliopsis. Science. 157:1564-1567.

Simon, R.C. 1963. Chromosome morphology and species evolution in the five North American species of Pacific salmon (Oncorhynchus). J. Morphol. 11277-97.

Simon, R.C. and R.E. Noble. 1968. Hybridization in Oncorhynchus (Salmonidae) I. Viability and inheritance in artificial crosses of chum and pink salmon. Trans. Am. Fish. Soc. 97(2):109-118.

Smith, L.T. and H.L. Lemoine. 1979. Colchicine induced polyploidy in brook trout. Prog. Fish Cult. 41(2):86-88.

Solar, I.I., E.M. Donaldson and G.A. Hunter. 1984. Induction of triploidy in rainbow trout (Salmo gairdneri Richardson) by heat shock and investigation of early growth. Aquaculture. 42:57-67.

Thorgaard, G.H. 1976. Robertsonian polymorphism and constitutive heterochromatin distribution of chromosomes of the rainbow trout (Salmo gairdneri). Cytogen. Cell. Genet. 17:174-184.

Thorgaard, G.H. and G.A.E. Gall. 1979. Adult triploids in a rainbow trout family. Genetics. 93:961-973.

Thorgaard, G.H., M.E. Jazwin and A. Stier. 1981. Polyploidy induced by heat shock in rainbow trout. Trans. Am. Fish Soc. 110:546-550.

Thorgaard, G.H., P.S. Rabinovitch, M.W. Shen, G.A.E. Gall, J. Propp and F.M. Utter. 1982. Triploid rainbow trout identified by flow cytometry. Aquaculture. 29:305-309.

Utter, F.M., O.W. Johnson, G.H. Thorgaard and P.S. Rabinovitch. 1983. Measurement and potential applications of induced triploidy in Pacific salmon. Aquaculture. 35:125-135.

Valenti, R.J. 1975. Induced polyploidy in Tilapia aurea (Steindachner) by means of temperature shock treatment. J. Fish. Biol. 7:519-528.

van Overbeeke, A.P. and J.R. McBride. 1971. Histological effects of 11-ketotestosterone, 17-α methyltestosterone, estradiol, estradiol cypionate, and cortisol on the interrenal tissue, thyroid gland, and pituitary gland of gonadectomized sockeye salmon (Oncorhynchus nerka). J. Fish. Res. Bd. Can. 28:477-484.

Van Tuinen, P. and M. Valentine. 1982. Mammalian chromosome newsletter. 23(4):182-185.

Wolters, W.R., C.L. Chrisman and G.S. Libey. 1981a. Lymphocyte culture for chromosomal analysis of channel catfish (Ictalurus punctatus). Copeia, p. 503-504.

Wolters, W.R., G.S. Libey and C.L. Chrisman. 1981b. Induction of triploidy in channel catfish. Trans. Am. Fish. Soc. 110:310-312.

Wolters, W.R., C.L. Chrisman and G.S. Libey. 1982c. Erythrocyte nuclear measurements of diploid and triploid channel catfish, (Ictalurus punctatus). J. Fish. Biol. 20:253-258.

Wolters, W.R., G.S. Libey and C.L. Chrisman. 1982d. Effect of triploidy on growth and gonad development of channel catfish. Trans. Am. Fish. Soc. 111:102-105.