DIPLOID AND TRIPLOID CHINOOK SALMON, *ONCORHYNCHUS TSHAWYTSCHA* (WALBAUM): AN EXPLORATION OF INDUCTION EFFICACY, PERFORMANCE AND GENOMIC ARCHITECTURE

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Abstract

Triploid salmon are sterile and thus may comprise part of an overall plan to minimize potential genetic disturbances to wild populations caused by the rearing of farmed fish in open seawater netpens. Despite the potential benefits of sterility, triploids are not widely reared for aquacultural purposes in North America mainly due to variable and inconsistent performance. While triploidization is being explored in an increasing number of species, the effect of triploidization in chinook salmon (*Oncorhynchus tshawytscha*) has rarely been investigated. In this study, chinook salmon were triploidized in order to assess 1) the efficacy of two triploid induction techniques 2) the utility of triploid chinook salmon for commercial aquaculture and 3) to develop a description of the genomic architecture of triploids. In Chapter One, a comparative examination of triploidization success and whole organism performance (survival, growth and the antibody response to vaccination) in diploid, heat-shock induced triploid and pressure-shock induced triploid full-sib family groups was carried out in terms of the effect of treatment, genotype (family) and treatment by genotype (family) interactions. In Chapter Two, a comparative examination of performance (survival, growth, and the lysozyme activity response to vaccination) in terms of the distribution and magnitude of phenotypic variance was carried out using a quantitative genetic framework and a paternal half-sib experimental design. Variance was partitioned into additive genetic and a combined epistasis, dominance and maternal effects component and narrow sense heritability values were calculated.

Minimal differences in triploidization success, growth and immune functioning were found between heat- and pressure-shock treated family groups. Although pressure-shock treated fish survived better than heat-shock induced triploids, triploids did not survive or grow as well as diploids. Survival of treatment groups was significantly

influenced by treatment and family effects while growth traits and antibody response to vaccination were more strongly influenced by the effect of family. Interaction effects were most prevalent for immune function.

Triploidization increased total phenotypic as well as additive genetic variance but this was associated with an unexpected and counter-intuitive decrease in the influence of the non-additive component (combined epistatic, dominance and maternal effects) indicating that triploidy may not have increased the genetic complexity of relationships among alleles or loci and that the primary effect of triploidization was additive and dominant. This was also highly suggestive of an overall ploidy dependent regulation of gene expression.

The obvious dichotomy between high and low performing families regardless of treatment/ploidy status, the existence of significant family components for many of the performance variables combined with increased heritability values for the measured traits indicated that selective breeding of diploids for increased triploid performance might be successful. However, the presence of family by treatment interactions (although explaining a relatively low amount of variance) observed in the Chapter One study, the increased range of phenotypic variance and profoundly different pattern of variance partitioning found in the Chapter Two study suggest that the production of a uniform fish product, at least during the freshwater period of growth might be compromised.

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General Introduction

Polyploidy, the state of having three or more complete sets of nuclear chromosomes, has recently received considerable attention in the fields of genetics, evolution and aquaculture. This interest is in part attributable to the usefulness of a polyploid platform for dissecting patterns of genetic expression and regulation and also because of the insights that might be gained by understanding the processes affecting the evolutionary fate of duplicated genes (Spring 1997; Galitski *et al.* 1999; Suzuki *et al.* 1999; Force 2000; Otto and Whitton 2000; Otto and Yong 2002). While polyploidy has long been known to have been a major evolutionary force involved in the diversification and adaptation of many plant species (Soltis and Soltis 1995; Ramsey and Schemske 1998) there is strong and accumulating evidence suggesting that two rounds of genome duplication occurred in the ancestral vertebrate lineage (Ohno 1970; Lundin 1993; Force 2000; Gibson and Spring 2000; Furlong and Holland 2002). Thus, polyploidy may have played an important role in defining both the rate and form of species diversification by providing the genetic structure and variation necessary for rapid adaptive evolutionary change (Ohno *et al.* 1968; Ramsey and Schemske 1998; Force *et al.* 1999; Otto and Whitton 2000).

Polyploidy is specifically known to have played an important role in the evolution of a number of fish families including the catfish *Corydoras-Aspidoras-Crochis* species group (within the family Callichthyidae), suckers (Catostomidae; Ferris and Whitt 1978; Ferris 1984) and the salmon and trout (Salmonidae; Schultz 1979; Allendorf and Thorgaard 1984). While tetraploidy in catostomids is thought to have arisen via hybridization (allopolyploidy) followed by a relatively rapid re-diploidization, salmonids exhibit evidence of intraspecific genomic doubling (autopolyploidy) followed by an incomplete transition back to the diploid state (e.g., residual tetrasomic inheritance and

the presence of multivalent pairing of chromosomes during meiosis) (Allendorf and Thorgaard 1984; Devlin and Nagahama 2002).

Salmonids provide intriguing models for the study of genetic change after polyploidization; however, because they are especially tolerant of many different kinds of chromosome set manipulations, including artificially induced polyploid states they also provide an interesting opportunity to study potential phenotypic or genomic effects of perturbation. Ploidy manipulation in fish has been studied since the 1950s, originally because of its usefulness in elucidating cytological function but also because of a perceived possibility for increased growth potential in salmonids reared for aquacultural purposes (reviewed by Ihssen *et al.* 1990). More recently, ploidy manipulation has received increased attention because of its applications to genetic mapping, studies of sex determination mechanisms, production of isogenic lines, inbreeding as well as whole organism performance (Ihssen et al 1990; Thorgaard 1992; Bongers *et al.* 1998; Arai 2001).

Ploidy manipulation to induce triploidy (the state of having three sets of chromosomes) has been of particular interest because it is relatively easy to induce in salmon and because of the potential for increased growth of fish reared for aquaculture. The production of monosex all-female triploid salmon is particularly useful in an aquacultural context because they are sterile. Sterility in female triploids is thought to be due to disrupted endocrinological functioning as well as problems with homologous chromosome pairing and segregation during gametogenesis which result in predominantly aneuploid germ cells (Benfey *et al.* 1989; Carrasco *et al.* 1998; Benfey 1999). Triploid females do not experience the typical sex steroid stimulation provided by the hypothalamus-pituitary-gonadal axis that diploids and triploid males respond to during maturation and so do not produce the vitellogenin and other factors necessary

for proper oocyte development or exhibit the gonadal growth, conditional degradation, sexual precocity or breeding behaviour normally experienced or displayed by diploids and triploid males (Benfey *et al.* 1986; Piferrer *et al.* 1994; Amano *et al.* 1998). This lack of maturation means that commercially-farmed fish may be grown on an extended schedule, potentially to larger sizes than diploids and harvested at any time. Perhaps more importantly, the lack of successful gametogenesis and spawning behavior means that all-female triploid fish farm escapees are unable to breed with wild stocks thus minimizing the potential threat that genetic introgression may pose for intraspecific diversity.

Along with sterility, all-female triploid salmonids exhibit morphological characteristics typical of an induced triploid state in vertebrates. In relation to diploids, these include: 1) nuclear enlargement with triploids containing 50% more DNA, 2) larger cell size with approximate maintenance of the nuclear:cytoplasmic ratio along with a reduction in the surface area:volume ratio, 3) fewer cells, but maintenance of diploid tissue, organ and body size and 4) increased allelic diversity with potentially three different alleles at each locus (Swarup 1959a; Leary *et al.* 1985; Benfey 1999). These conditions suggest that triploids may experience potentially positive fitness or production related effects due to increased allelic diversity, higher genetic expression of growth related loci and somatic reallocation of energy normally invested in the maturational process. While investigation of the physiological consequences of induced triploidy in fish is incomplete, especially at the cytological and genetic levels of study, exploration of physiological parameters such as hematology, oxygen consumption and aerobic capacity, a limited number of immunocompetence determinants, osmoregulation and stress response, energetics and development indicate either relatively subtle differences (e.g., intolerance of hypoxic conditions, Ojolick *et al.* 1995;

reduced sensory perception, Aliah *et al.* 1990) or an overall similarity of function rather than overt differences (reviewed by Benfey 1999).

There is a growing body of published work comparing the performance of diploids and triploids in terms of survival and growth in an increasingly diverse array of fish species (e.g. Benfey 1989; Pandian and Koteeswaran 1998; Felip *et al.* 2001; Arai 2001). Despite an overall trend indicating that triploids usually experience lower survival than diploids especially during the juvenile stages of development (Solar *et al.* 1984; Happe *et al.* 1988), effects of triploidization on growth tend to be more variable between studies with results including growth less than, greater than and equal to that of diploid fish.

Despite similarities between diploids and triploids in physiological parameters and the variability of triploid performance, direct comparison of studies is often confounded by differences in study design and conditions (including strain, sex, age, size and genetic origin of fish (Benfey 1999) and possibly induction method) as well as potential species-specific differences in the tolerance of or response to triploidization (Thorgaard 1986; Ihssen *et al.* 1990). Additionally, very few studies have focused on Pacific salmon species such as chinook salmon (*Oncorhynchus tshawytscha*).

Triploid salmon can be produced by breeding tetraploids with diploids (e.g.,Chourrout *et al.* 1986) but are more commonly induced by applying a short thermal or pressure-based shock to fertilized ova (Ihssen et al 1990; Felip, Zanuy, Carrillo, and Piferrer 2001; Hulata 2001). Because salmon eggs are ovulated after completion of meiosis I and in a state of arrested metaphase II, only the second meiotic division is available for manipulation. Fertilization triggers a meiotic reactivation cascade that facilitates the transition to anaphase (Holloway *et al.* 1993; Ciosk *et al.* 1998). If a shock is applied to the egg shortly after fertilization but before completion of anaphase II, the

haploid chromosome set normally extruded as the second polar body can be retained within the egg cytoplasm. If fusion of this chromosome set with the maternal and paternally contributed chromosome sets occurs prior to the first mitotic cleavage, the embryo will develop as a triploid.

Interestingly, the specific cytological mechanisms by which heat and pressure treatments cause second polar body retention have not been examined in detail, despite evidence to suggest that the meiotic response to heat and pressure may differ (e.g., Fankhauser and Godwin 1948; Swarup 1959b; Chourrout 1986). It is also not known if heat and pressure shock-treatments applied to fertilized fish eggs affect subsequent embryonic development or cellular functioning in a differential manner or whether there are lasting effects of treatment on cellular or organismal performance (however, see Malison *et al.* 1993). This may be partly due to a strong research focus on production-based goals (primarily triploidization success and growth) and difficulty in separating treatment effects from the effects of triploidization *per se*. Additionally, there has been no published work investigating potential phenotypic effects on quantitative genetic parameters after triploidization in salmon.

The current research includes two quite different, but interrelated, perspectives. The first study, as outlined in Chapter One, has a very applied aquacultural focus and explores the efficacy of triploidization methods as well as the comparative freshwater performance of diploid and triploid chinook salmon full-sib families generated using heat and pressure-shock methodologies. The impact of triploidization on performance was examined in terms of the effects of genotype (family), treatment and genotype by treatment interactions on growth and immune characteristics. The study outlined in Chapter Two has a less applied focus but extends the range of Chapter One by exploring the effect of triploidization on both the phenotypic variance and the additive

genetic control of specific performance variables using a quantitative genetic analysis framework.

CHAPTER 1

COMPARISONS AMONG DIPLOID, HEAT-SHOCK INDUCED TRIPLOID AND PRESSURE-SHOCK INDUCED TRIPLOID CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) FOR SURVIVAL, GROWTH, IMMUNE FUNCTION AND TRIPLOIDIZATION EFFICACY

1.0. Abstract

The production of sterile salmon for aquaculture would be an effective strategy with which to minimize potential genetic risks associated with fish farm escapes. All-female triploid salmon are sterile and can be relatively easily generated by applying a heat or pressure-based shock to eggs. However, while triploidization in fish has been examined for some time, this technology has not been widely implemented in North American fish farms. This is mainly due to inconsistent or inferior performance of triploids as compared to diploids but there is also a lack of species-specific research especially for chinook salmon (*Oncorhynchus tshawytscha*). Additionally, the comparative efficacy of heat and pressure-based induction protocols and the effect of treatment and genotype on fitness related traits have rarely been examined or controlled for in the same study, despite strong evidence indicating that these are important variables.

In this study, the effect of treatment and genotype on freshwater survival, growth, immune function and triploidization success was examined in full-sib groups of heat- and pressure-shock induced chinook salmon triploids and diploids originating from each of five families. It was found that triploidization rates were highest for pressure-based induction, but few differences between the two treatment groups for survival, growth and antibody response to intraperitoneal vibrio (*Vibrio anguillarum*) vaccination were observed. While triploids had lower survival and poorer performance than diploids,

significant differences occurred mainly during the embryonic or larval stages of development. Survival was mainly influenced by treatment (heat-shock, pressure-shock) and family (genotype) while growth characteristics and the immune response to vaccination were mainly influenced by family (genotype). Significant interactions between family (genotype) and treatment were found. This interaction explained a relatively low proportion of the total variation in survival and growth but a large proportion of the variation in immune response. Despite the occurrence of interactions, separation of high and low performing families was evident regardless of ploidy and family effects were substantial indicating that selection of high performance diploid broodstock for triploidization purposes would be part of an effective strategy for improvement of triploid offspring performance.

1.1. Introduction

Teleosts are tolerant of a wide range of chromosomal manipulations and artificially induced polyploid states (e.g., see Arai 2001 1997; Pandian and Koteeswaran 1998). Triploidy (the genomic state of having three complete sets of chromosomes) is especially easy to induce and salmonids appear to be quite tolerant of this condition (Thorgaard 1992; Benfey 1991; Ihssen *et al.* 1990). All-female triploid salmon are of particular interest because they are sterile and so may have considerable economic and environmental benefits for aquaculture (Cotter *et al.* 2000; Wilkins *et al.* 2001).

Some potential advantages of using sterile salmon for aquaculture include: 1) the circumvention of sexual maturation and associated conditional degradation, 2) increased somatic growth, 3) extended grow-out, 4) wider harvest windows and 5) minimization of possible genetic and ecological threats to wild populations. The technology, however, has not been widely implemented in North American salmon farms. This is predominantly due to inconsistent or highly variable performance-based results, and a general trend indicating that triploids usually experience lower survival than diploids and may experience reduced growth (Benfey 1999). The precise causes of impaired performance are not known [but may include treatment induced stress effects (e.g., Swarup 1959; Malison *et al.* 1993) and ploidy related differences in cellular level physiology (Benfey 1999), or genetic disruptions]. These factors may be most important to triploid survival during the critical stages of emybryonic development. Despite growing evidence indicating the importance of species and family-specific genetic differences in the tolerance of triploidization in salmon (see Withler *et al.* 1995; Withler *et al.* 1998; Bonnet *et al.* 1999; Blanc *et al.* 2001), direct comparisons across published performance-based studies are often confounded by differences in study

conditions (i.e., strain, sex, age, size; Benfey 1999) or a lack of species or population-specific induction protocol optimization.

Triploidization of all-female salmon is most commonly and successfully induced by applying either a heat or pressure-based shock to ova fertilized with sperm from hormonally sex-reversed females (i.e., neomales). It is possible that the trauma associated with these treatments may be contributing to the impaired performance of triploids; however, neither the specific treatment effects associated with the application of heat and pressure, nor the potential for long-term effects of treatment on cytological, genetic and molecular aspects have been investigated, and the effects of triploidy *per se* on these parameters as well as whole organism functioning has only been investigated very superficially in fish (i.e., primarily in terms of traits that are of economic importance to aquaculture).

Suggestions that heat and pressure may have different effects on both amphibian and fish oocyte meiotic and mitotic structure and overall functioning exist in the literature (e.g., Fankhauser and Godwin 1948; Swarup 1959; Chourrout 1986; Diter *et al.* 1993). It is known that heat and hydrostatic pressure can have severe structural effects on microtubule and cytoskeletal structural integrity; however, these effects may be reversible (Wilson *et al.* 2001b; Begg *et al.* 1983). Disruption of microtubule structure during meiosis is thought to be the mechanism by which the second polar body is forced to remain in the egg after triploidization treatment (however, see Fankhauser and Godwin 1948); heat and pressure may also have other effects on eggs subjected to triploidization treatments. Pressure is known to disrupt protein structure and important cellular regulatory processes as well as change protein and mRNA distribution patterns (Begg, Salmon, and Hyatt 1983; Crenshaw *et al.* 1996; Wilson *et al.* 2001a; Wilson, Zimmerman, and Zimmerman 2001b). Heat tends to have acute effects on cell

structures such as membranes (fluidity) and proteins (denaturation) but may also result in critical impairments of cell and genetic functioning (Hildebrandt *et al.* 2002). While applications of heat and pressure to oocytes have the potential to seriously interfere with subsequent embryonic development and cellular functioning, application at the intensity and duration used to triploidize fish is probably not severe enough to cause acute structural damage to the activated oocyte but might interfere with subsequent cellular expression patterns and protein function.

While identifying the least traumatic method for triploidization is important, the adequate performance of triploids under conditions of culture is a necessary requirement for the adoption of triploid technology by the aquaculture industry. There is also a need to determine if increases in performance gained by using traditional selective breeding programs and molecular-based selection techniques can be transferred to fish after triploidization. As part of this evaluative process, I generated full-sib chinook salmon (*Oncorhynchus tshawytscha*) family groups consisting of heat-induced triploids, pressure-induced triploids, and diploid controls. In this way I was able to determine which of the two induction methods was most effective for triploidizing chinook salmon while comparing the effects of treatment, family (genotype) and the interaction between treatment and family (treatment x family) on specific fitness-related performance characteristics during freshwater rearing. I chose to follow the family treatment groups only through the freshwater stages of development because the early life history of salmon consists of a series of critical stages when selective pressures are intense and at which family, treatment and family by treatment interaction effects on performance variables might be most evident. Inferior performance during the critical stages of juvenile development can potentially have long-term negative effects on overall fish performance (Rossiter 1998). The goal of this study was therefore to

supplement existing literature evaluating the utility of triploids for aquacultural purposes by providing clear evidence on the impact of treatment and genotype on triploid chinook salmon performance throughout the freshwater phase of development.

1.2 Methods

1.2.1 Fish & Rearing Conditions

Mature monosex female chinook salmon from Yellow Island Aquaculture, Ltd. (YIAL; Quadra Island, B.C.) were used to generate the offspring families for this study. Five phenotypic males (hormonally masculinized genotypic females) were bred 1:1 with 5 females to form five full-sib families. Spawning of all broodfish took place at YIAL hatchery facilities on Quadra Island, between October 31 and November 5, 2000. Broodstock were maintained in tanks supplied with hatchery water (mean water temperature ± SE = 8.05 °C ± 0.01 °C) prior to collection of gametes. Eggs from each full-sib cross were collected and dry fertilized separately. Time was tracked immediately upon addition of milt to the egg masses. The egg-milt mixture was left for 2 minutes to allow for fertilization before hatchery water at 8.05 °C ± 0.01 °C was added to induce swelling and micropyle closure and to wash excess milt and debris from the eggs. Three ~250 ml sub-samples of eggs from each fertilized egg mass were randomly sampled and subjected to one of the following three treatments: i) *Hydrostatic pressure-shock,* five minutes at 6.89 x 10^4 kPa (10 000 psi) of pressure, 30 minutes after fertilization (mean water temperature ± SE = 8.05 °C ± 0.01 °C). Pressure was applied using a modified 30-ton H-frame hydraulic press and a custom built 1.6 L experimental egg pressure cylinder ii) *Heat-shock,* ten minutes submerged in a 29.0 ± 1.0 ° C uniformly heated, aerated waterbath, 25 minutes after fertilization followed by 30 minutes of air-cooling. Eggs were placed in plastic mesh incubation boxes (Viberg) for the heat

treatment. Boxes were then hung on hooks in the hatchery for 30 minutes of air-cooling (mean air temperature ± SE = 8.4 °C ± 0.3 °C) iii) *Control.* Fertilized egg sub-samples from each female were left untreated and transferred to incubation trays immediately after the water hardening process. The particular treatment protocols used were based on extensive experimentation carried out by YIAL to optimize triploidization protocols for chinook salmon under the specific hatchery conditions at their facilities (YIAL has been experimenting with triploidization since 1993), and on a survey of treatment protocols designed for salmonid induction outlined in the literature (e.g., Hill, Hickerson *et al.* 1982; Benfey and Sutterlin 1984; Guoxiong, Solar *et al.* 1989).

Family treatment groups were incubated in separate compartments of vertical stack incubation trays (Heath Tecna Corp.). Trays were divided into twelve compartments (10 cm x 10 cm x 5cm) with each compartment holding an average of 700 eggs. Offspring families were assigned to incubation stacks and trays randomly. Water temperature within the stacks was monitored using a digital data logger (Onset Computer Corp.) and development stage of the fish was tracked using Accumulated Temperature Units (ATUs; calculated as the cumulative total of daily mean temperatures). Mean water temperature (± SE) during the incubation period was 7.72 °C ± 0.02 °C. Mean flow within the stacks was 13 L/minute.

When eggs reached the eyed stage of development (the point at which the eye spots of the developing embryo are visible through the egg shell and at which ¾ yolk vascularization has occurred; November 28-December 11, 2000, 280-296 ATUs) they were mechanically shocked and sorted using a Jensorter machine (Model JM4C, Jensorter, Inc.) in order to get total egg counts and to separate live from dead eggs within each family treatment group.

As alevins completed yolk-sac absorbtion (March 7-11, 2001; 973-982 ATUs),

two sets of fifty alevins each were randomly selected from the treatment groups within each of the five families (two replicate groups from each family treatment groups) and transferred from the vertical incubation stack tray compartments to 140 L aerated rearing tanks for the onset of exogenous feeding. Two sets of fish were randomly assigned to each tank regardless of ploidy at a starting density of 100 fish/tank (~0.71 fish/L; mean density = 0.27g/L). One set of fish in each tank was fin clipped for identification purposes (either the upper or lower caudal fin lobe was removed). Flow rate of water to the tanks was approximately 3 L/minute and the mean temperature (± SE) was 8.79 °C ± 0.02 °C. Fish were handfed to satiation multiple times per day with commercial feed (Ewos, Canada, Ltd.).

1.2.2. Ploidy Determination

Two techniques, flow cytometry and red blood cell (rbc) nuclear analysis, were used to determine the level of triploidization success within heat and pressure treated groups of fish. Both methods are commonly used to determine the ploidy level of fish subjected to triploidzation treatments. While flow cytometry is a highly sensitive method and allows accurate measurement of the double-stranded DNA content of individual cells or nuclei, the measurement of rbc nuclear dimensions is strongly and positively correlated with genome size (C-value) in fish (Gregory and Hebert 1999), is simple and does not require expensive equipment. Both methods are able to reliably distinguish between triploid and diploid salmonids (e.g., Allen 1983; Benfey *et al.* 1984; Small and Benfey 1987; Johnson *et al.* 1984; Teplitz *et al.* 1994). Flow cytometry was used in this study to verify the results of the rbc nuclear analysis.

Erythrocyte nuclear length was used to determine family-specific triploidization success (Wolters *et al.* 1982; Beck and Biggers 1983; Benfey, Sutterlin, and Thompson

1984). Whole blood smears were made from all terminally sampled fish. Slides were fixed in methanol and stained with Wright-Giemsa (Sigma). Visualization and measurement of erythrocyte nuclei was accomplished under oil immersion (1000x magnification) using an Olympus BX-50 compound microscope (Olympus Optical Co.) equipped with a QImaging Retiga 1300 Monochromatic digital camera (Quantitative Imaging Corp.) and the Northern Eclipse, version 6.0 imaging program (Empix Imaging Inc.). The length of ten randomly chosen erythrocyte nuclei was measured to the nearest 0.01μm in each of 778 whole blood smears sampled from heat (n = 230), pressure (n = 245) and control (n = 303) treatment groups within each of the five study families.

Two hundred fish (twenty fish per family treatment group) from the five study familes were also terminally sacrificed at 186-200 days post-fertilization for flow cytometric analysis. Blood samples were prepared using a modified version of Allen's (Allen 1983) protocol (G. Osbourne, UBC Biomedical Research Centre). Briefly, 1.5-5.0 μl of blood was collected from the caudal vasculature of each fish and ejected into 0.5 mL of a 50 mg/L solution of propidium idodide (pH 7.2; 50 mg propidium iodide, 25 ml citrate acid dextrose, 8.5 mg RNAse A, 1.0 ml IGEPAL-630, phosphate buffered saline to 1 L) kept on ice. Each sample was vortexed and refrigerated at 4 °C overnight to allow membrane disruption and intercalcation of the propidium iodide dye with dsDNA. In the morning, 4% paraformaldahyde solution was added to each sample. Samples were kept on ice and transported to the Multiuser Flow Cytometry facility in the Biomedical Research Centre at the University of British Columbia for analysis on the FACSCAN (Becton Dickinson) flow cytometer. Blood sampled from 10 diploid control fish was used as a series of external standards and DNA indexes [modal DNA content (channel #) of test sample /modal DNA content (channel #) diploid standard] were

calculated to determine ploidy status.

1.2.3. Survival

Incubation survival of family treatment groups was monitored from fertilization to the eyed stage of development (280-296 ATUs) and then followed through to the alevin stage, just prior to transfer of the fish to freshwater rearing tanks (mean ATUs = 970). Embryo mortalities from fertilization to the eyed stage (3/4 yolk vascularization) were assessed after the eggs were mechanically shocked and sorted using a Jensorter machine. Total egg number and the number of live eggs for each family treatment group were evaluated at this time. After the initial eyed egg count, mortality was monitored at least every two days. Survival (as a proportion of the initial live eyed egg count) was determined for all groups at 510 ATUs (post-hatch), 616 ATUs, 746 ATUs, 789 ATUs, 853 ATUs, and 970 ATUs (just prior to ponding).

Survival of the five experimental families (i.e., heat, pressure and control treatment groups from each family plus replicates totaling 30 treatment groups) was monitored at least every second day after transfer to rearing tanks. Survival was determined as the number of live fish remaining immediately prior to experimental vaccination treatment divided by the total number of fish originally fin-clipped and released into the rearing tank. Experimentally sacrificed fish (i.e. for flow cytometry) within each group were excluded from the final survival assessment.

1.2.4 Growth

Weight (in grams) was determined by non-terminal sampling at five developmental time points during the freshwater growth of the ponded family groups, at ponding (mean ATUs = 985; ~127 days post-fertilization), at 1 200 ATUs (mean ATUs =

1224; ~155 days post-fert.), at 1 700 ATUs (mean ATUs = 1729; ~214 days post-fert.), at 1 800 ATUs (mean ATUs = 1843; ~227 days post-fert), and at 1 900 ATUs (mean ATUs = 1941; ~236 days post-fert.). At ponding, 100 alevins from each family treatment group were weighed in water as they were transferred to rearing tanks. At all other sample times, weights of individual fry were recorded. Forty fish per family treatment group (heat, pressure, control + replicates) were weighed at the 1 200 and 1 700 ATU sample points. At the1 800 and 1 900 ATU sample points, approximately 66 fish were sampled from each treatment group (treatment group and replicate) within all families. Blood smears for ploidy determination were taken from all fish sampled at the 1 800 ATU and 1 900 ATU sample points but not at the 1 200 and 1 700 ATU sample points.

Relative growth rate of family treatment groups was assessed from ponding to 1 900 ATUs (mean ATUs = 985 - 1941), an average time interval of 112 days. Because individual fish were not tracked, mean family treatment group weight at ponding was used for the first sampling point and individual fish weights were used for the second sampling point. Relative growth rate was calculated using:

$$\left\{\left(Y_2 - \overline{Y_1}\right) \div \overline{Y_1}\left(t_2 - t_1\right)\right\} \times 100$$

Where Y_2 = individual fish weight at the second sampling point; $\overline{Y_1}$ = mean family treatment group weight at ponding, $t_1 - t_2$ = the mean time interval in days between the first and second sampling points.

1.2.5. Vaccination & Enzyme Linked Immunosorbent Assay (ELISA)

To determine if treatment groups differed in their serum antibody response to vaccination, a vaccination trial using four of the original five families was conducted. Within a family, each fish within one replicate treatment group received a 0.1 mL dose

of a commercial water-based vibrio vaccine (Alpha Dip 2100, Alpharma NW Inc.; *Vibrio anguillarum*, serotype 01 and *V. ordalii* bacterin) by intraperitoneal injection. Each fish in the second family treatment replicate group received 0.1 mL of a phosphate buffered saline solution. All fish within each treatment group were anaesthetized, weighed, injected and returned to the appropriate rearing tanks for ten days to allow for establishment of an antibody response. Mean treatment group weights at the time of injection were: heat = 3.91 g, pressure = 4.19 g, control = 4.58 g; the mean temperature at injection was 9.53 °C and ranged from 9.19 - 9.53 °C. On the eleventh day after vaccination, all fish were euthanized and weighed. Blood was taken from the caudal vasculature with uncoated microhematocrit tubes and a blood smear was made for ploidy determination via red blood cell analysis. Serum was transferred to microcentrufuge tubes after overnight refrigeration (4 °C) and centrifugation (5 minutes at 7100 rpm, 5125 x g). All serum samples were held at –20 °C and then at –80 °C until determination of antibody titre by enzyme linked immunoassay (ELISA). Two hundred diploid YIAL production fish (mean weight at injection = 5.80 g) were used to generate a positive control serum for use as a control standard in the immunoassay. Fish were injected with the Alpha Dip 2100 vaccine, as noted above and were terminally sampled on the 11th day after vaccination (temperature at injection = 8.93 °C; mean temperature over serum generation period = 9.29 °C, range = 8.93 - 9.76 °C).

Specific antibodies against *Vibrio anguillarum* were detected in fish sera after vaccination using an indirect ELISA protocol (R. Beecroft, Immuno-Precise Antibodies, Ltd.). Microtitre plates (96 wells; Nunc Maxi Sorb) were coated with a *Vibrio anguillarum* cell suspension (culture provided by G. Prosperi-Porta, Pacific Biological Station, Nanaimo, BC). Plates were incubated overnight at 37 °C and blocked with a 3% normal

goat serum phosphate buffered saline solution. Salmon anti-vibrio immune serum was diluted 1/20-1/2560 across the plates. Four salmon anti-vibrio serum samples as well as positive (pooled control serum), negative (family treatment group specific negative control serum-sham injected) and blank controls were run on each plate. Rabbit anti-salmon immunoglobulin (H+L chain; ImmunoPrecise Antibodies, Inc.; 1/4000) 2 ° antibody and goat anti-rabbit iummunoglobulin G (H + L), 3 ° antibody labeled with alkaline phosphatase (Caltag, Inc.; 1/2000) were used for antibody detection. Alkaline phosphatase substrate solution was added and the optical density ($OD_{405\ nm}$) determined after 30 minutes using a VERSAmax tunable plate reader with Softmax Pro 4.0 software (Molecular Devices, Corp.). Four salmon anti-vibrio immune serum samples from each of the three family treatment groups within four of the five study families as well as negative control serum samples (sham-injected) from each family treatment group were analyzed by ELISA.

1.2.6 Statistical Analysis

Data normality was assessed visually and with normal probability plots. Hartley's F_{max} test (Hartley, 1940, 1950) was used to assess homogeneity of variance among treatment types for each measurement of performance. Data transformation was used if data non-normality or variance differences were extreme. Generally, two-way mixed model analysis of variance (ANOVA) was used to detect treatment (fixed effect), family (random) and the treatment by family interaction effects on performance measurements. Multiple comparisons using Tukey's Honest Significant Difference (HSD) test were performed when significant effects ($P < 0.05$) were detected. Reaction norms were plotted to visually clarify and interpret significant interactions and effect sizes. Effect size of the main factors and the interaction was also determined using omega squared (ω^2,

proportion of total variance). Omega squared was calculated using the formulas of Dodd and Schultz (1973) for fixed, random and interaction effects (reported in Olejnik and Algina 2000) and Cohen's (1992) scale of effect size (i.e., $0.0099 \leq$ small < 0.0588; $0.0588 \leq$ medium < 0.1379; large ≥ 0.1379). One-way ANOVA was used to detect treatment effects at the eyed stage of development, tank or incubation position effects or the effect of tank ploidy composition (diploid, triploid or mixed) on measured variables even though placement of family groups was completely randomized. Analyses were performed using Systat Version 10.0 (SPSS Inc. 2000) and SPSS Version 10.0.1 (SPSS Inc. 1999).

1.3 Results

1.3.1 Triploidization Success

Overall triploidization success levels measured using red blood cell nuclear analysis and flow cytometry were high for both heat- and pressure-shock treatments. Flow cytometry data confirmed the results obtained by the red blood cell nuclear analysis. Ninety-seven percent of both heat and pressure-shock treated fish were found to be triploid by flow cytometry, while rbc nuclear analysis indicated that overall triploid levels were 94% for heat-shock treatment groups and 96% for pressure-shock treatment groups; all sampled control fish were identified as diploid. There was some unexpected overlap between the diploid and triploid nuclear length distributions (Figure 1.1). Therefore samples with mean rbc nuclear lengths between 8.25 μm and 8.75 μm (between dotted lines in Figure 1.1; n = 8 or 1.0% of sampled fish) were eliminated from the analysis because of uncertainty in ploidy designation. It is possible that these fish were aneuploid but this could not be established as blood was not taken for flow cytometric analysis from these specific fish. Aneuploid salmon do not usually survive

very long past hatching (Chourrout 1986) and no evidence of aneuploidy was detected in the samples analyzed with flow cytometry. The overlap between measurements may reflect natural variability in rbc nuclear size. Red blood cell nuclear length was significantly different between treatment groups ($P < 0.001$). Diploid control groups had significantly smaller rbc nuclear lengths than pressure and heat-shock groups ($P < 0.001$) however, pressure and heat-shock groups did not differ significantly from each other ($P = 0.89$; mean rbc nuclear length $\pm$ SD, 2N-Control = 7.33 μm $\pm$ 0.38 μm; 3N-Heat = 9.32 μm $\pm$ 0.54 μm; 3N-Pressure = 9.30 μm $\pm$ 0.51 μm).

While pressure-shock treatment was more successful than heat-shock treatment at inducing triploidization according to the rbc analysis (mean $\pm$ SD, 3N-Heat = 0.94 $\pm$ 0.03, 3N-Pressure = 0.96 $\pm$ 0.04), the difference between treatment success rates was small (2%) and not significant. Results from the mixed model ANOVA using family triploidization proportions (arcsin$\sqrt{}$ transformed) indicated that the main effects of treatment and family were not significant (Table 1.1) but there was a large and statistically significant interaction ($P < 0.001$), indicating that triploidization levels varied across families dependent on the nature of the applied treatment (Figure 1.2). The magnitude of the effect size of the interaction was large (Cohen 1977) (i.e., the proportion of the total variation in triploidization success that was explained by the interaction was high; refer to omega squared values ω^2, Table 1.1) however, the overall family-based level of variation in triploidization success was low for both treatments (89%-100%) suggesting that differences in the efficacy of treatment were not large.

Table 1.1. Analysis of variance components and omega squared values (ω^2) for red blood cell nuclear analysis.

Source of Variation	Sum-of-Squares	df	Mean-Square	F-ratio	ω^2
Treatment	0.0558	1	0.05576	2.357	0.02
Family	0.1140	4	0.02854	1.206	0.31
Treatment x Family	0.0095	4	0.02366	1217.465***	0.53
Residual	0.0002	10	0.00002		

*** indicates significance at the P < 0.001 level

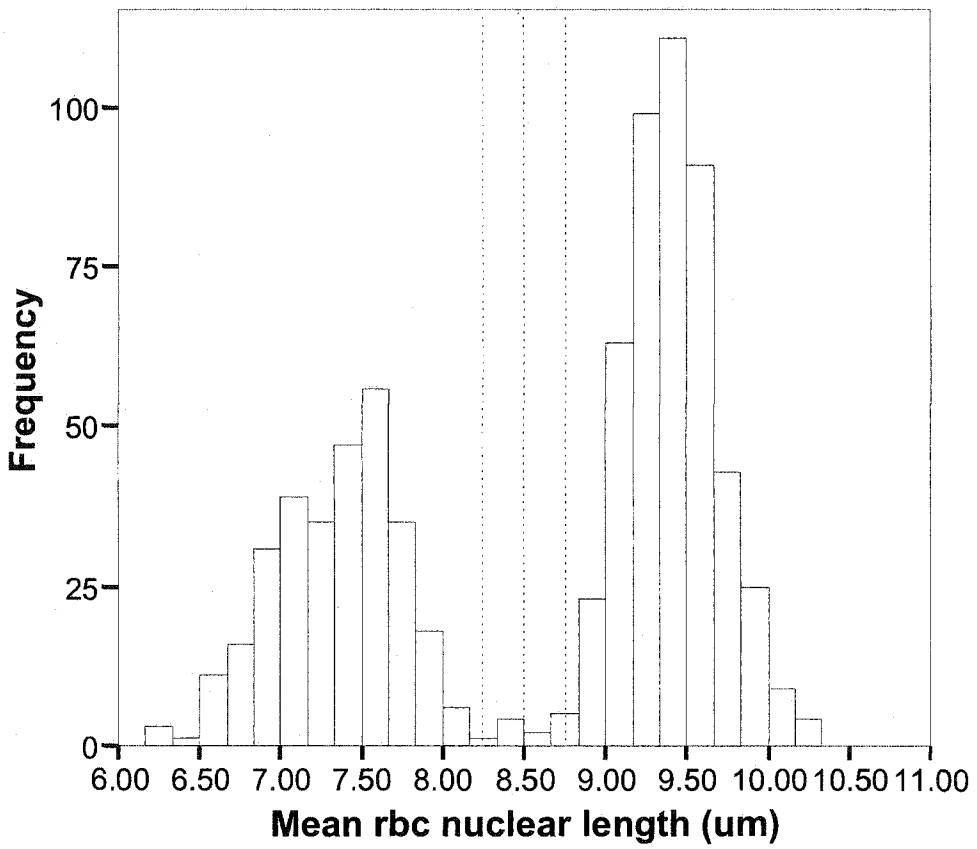


Figure 1.1. Frequency distribution of mean red blood cell nuclear length across treatment groups. Nuclei > 8.5 μm = 3N, nuclei < 8.5 μm = 2N. Samples with mean rbc nuclear length between 8.25 μm and 8.75 μm (between dotted lines; n = 8, 1.0% of sampled fish) were eliminated from the analysis because of uncertainty in ploidy designation.

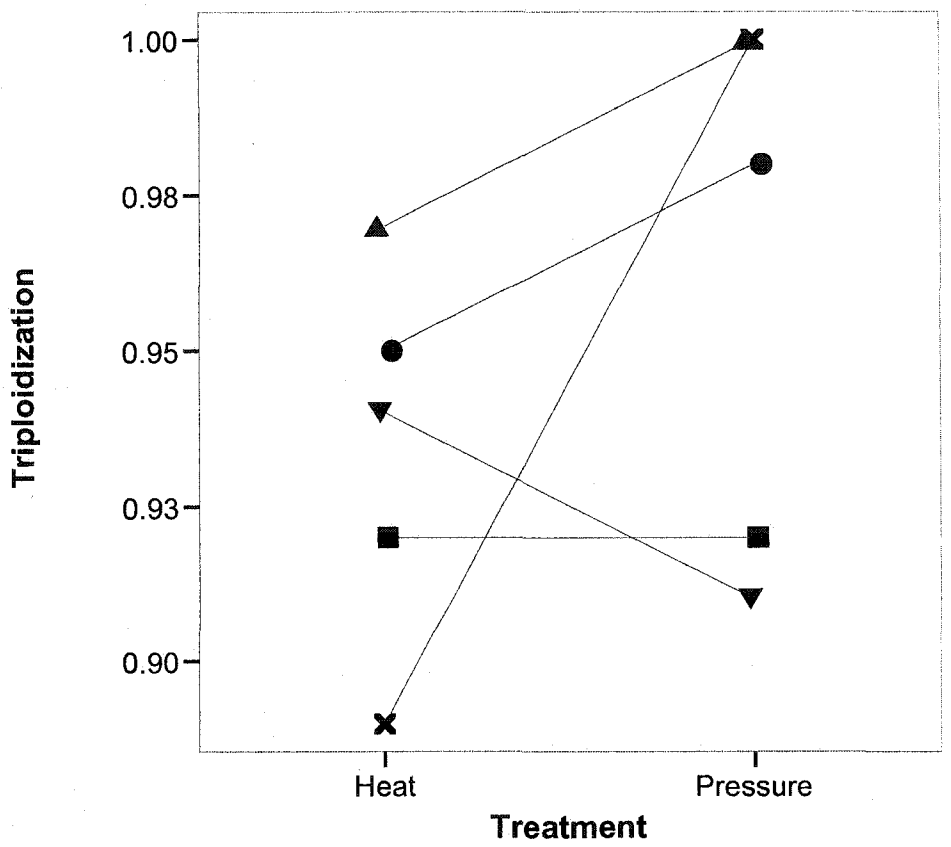


Figure 1.2. Reaction norms for family effects on mean treatment group triploidization success levels. Triploidization success levels were determined using the length of 10 red blood cell nuclei for each sampled fish (heat = 230 fish; pressure = 245 fish). Symbols identify family treatment groups.

1.3.2 Incubation Survival

A one-way ANOVA was used to test for the effect of treatment on survival (arcsin $\sqrt{}$ transformed) to the eyed stage of development. Treatment groups differed significantly in survival to this stage of development (~288 ATUs; P = 0.031) and a large proportion of the variation in survival was associated with the treatment effect (Table 1.2). Mean survival of the two triploid groups did not differ significantly (P = 0.997).

Diploids experienced the highest mean survival, but diploid mean survival was only significantly higher than that of the heat-shock treated triploids (P = 0.047) (mean back transformed values ± SD: 2N-Control = 0.95 ± 0.09; 3N-Pressure = 0.68 ± 0.12, 3N-Heat = 0.67 ± 0.02).

Table 1.2. Analysis of variance components and omega squared value (ω^2) for survival to the eyed stage of development.

Source of Variation	Sum-of-Squares	df	Mean-Square	F-ratio	ω^2
Treatment	1573.312	2	786.656	4.732*	0.34
Residual	1994.816	12	166.235		

*indicates significance at the P < 0.05 level

The overall magnitude of the effect of triploidization on survival (arcsin √ transformed) through the rest of incubation (after the eyed stage of development) was large and clearly negative with heat and pressure-shock treatment groups experiencing overall mean survival to the end of incubation that was 42% and 32%, respectively, below that of diploid control groups (mean back transformed values ± SD: 2N-Control = 0.83 ± 0.01; 3N-Pressure = 0.51 ± 0.03; 3N-Heat = 0.41 ± 0.01). The mean survival of the two triploidized groups (heat and pressure) did not differ significantly from each

Table 1.3. Analysis of variance components for survival after the eyed stage of development to the end of incubation.

Source of Variation	Sum-of-Squares	df	Mean-Square	F-ratio	ω^2
Treatment	7785.680	2	3892.840	11.067**	0.32
Family	5497.017	4	1374.254	3.907*	0.30
Treatment x Family	2814.084	8	351.761	8.231***	0.19
Residual	1.058	75	0.014		

*indicates significance at the P < 0.05 level; ** indicates significance at the P < 0.005; *** indicates significance at the P < 0.001 level;

other at any developmental stage during incubation at which survival was determined (i.e., at 510 ATUs , P = 0.971; 616 ATUs , P = 0.769; 746 ATUs, P = 0.812; 789 ATUs , P = 0.794; 853 ATUs , P = 0.866; 970 ATUs, P = 0.478; Figure 1.3). The mean survival

of heat-shock treatment groups was always significantly lower than that of control groups (except just after hatching, at 510 ATUs when the mean survival of heat, pressure and control groups did not differ significantly from each other, $P = 0.064$) (616 ATUs , $P = 0.041$; 746 ATUs, $P = 0.030$; 789 ATUs , $P = 0.032$; 853 ATUs , $P = 0.030$; 970 ATUs, $P < 0.001$) the mean survival of pressure-shock treatment groups was only significantly lower than the mean diploid control group survival at the end of incubation, just prior to ponding (970 ATUs; $P = 0.020$; Figure 1.3).

A significant interaction between family and treatment on incubation survival (Table 1.3) was found to exist but this interaction only described 19% of the total variance in survival (ω^2, Table 1.3). To describe the interaction, reaction norms showing the effect of family of origin on treatment group survival at each developmental stage were plotted (Figure 1.4). The graphs clearly show that the response to treatment varies depending on family of origin and that treatment and family effects cannot be interpreted without considering this interaction. Interestingly, an obvious distinction can be seen between families that survived relatively well regardless of applied treatment (i.e., high performance families) and those that survived poorly as triploids (heat-shock or pressure-shock); however, this distinction appears to become less important by the end of incubation (970 ATUs) when treatment effects tend to dominate (Figure 1.4 f). The effect size of all factors (main and interaction) was large but the treatment and family effects explain more of the variation in *overall* survival than does their interaction (i.e., just prior to ponding at 970 ATUs, Figure 1.4f; Table 1.3, ω^2 values). Survival was arcsine transformed to attain normality for analysis; reported means and SDs are back-transformed values. Interestingly, there appears to be more variation in survival in response to pressure- than to heat shock treatments. There were no significant effects of the section position within treatment groups within the incubation trays on survival at

any developmental stage during incubation (P > 0.05).

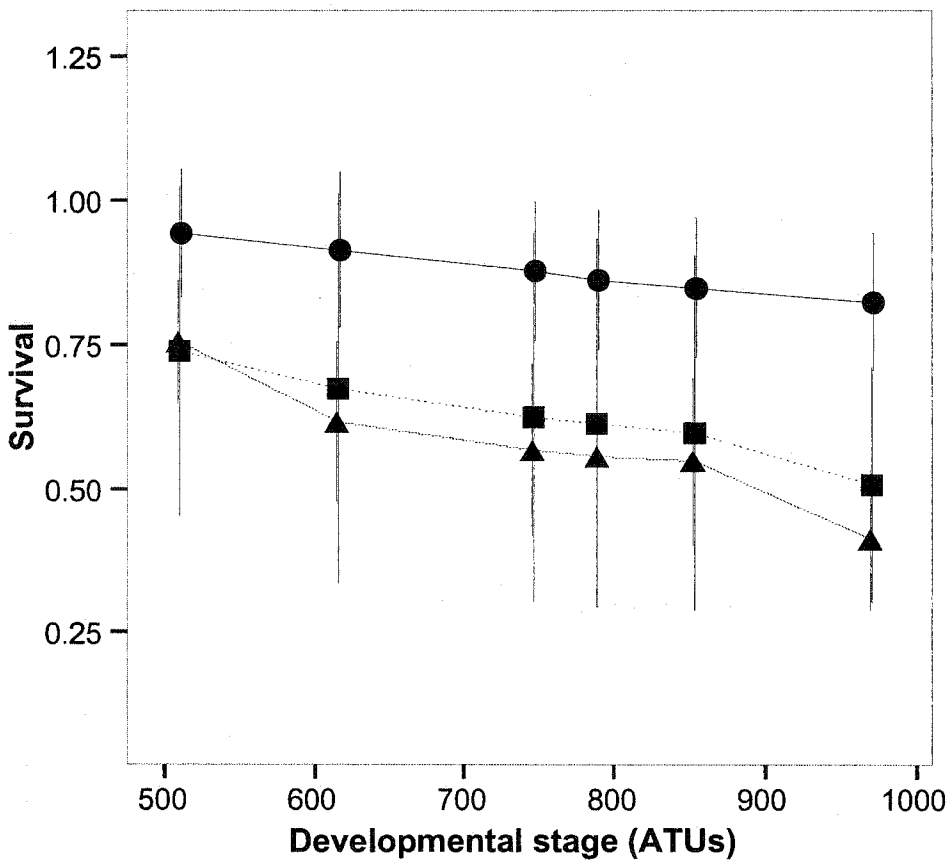
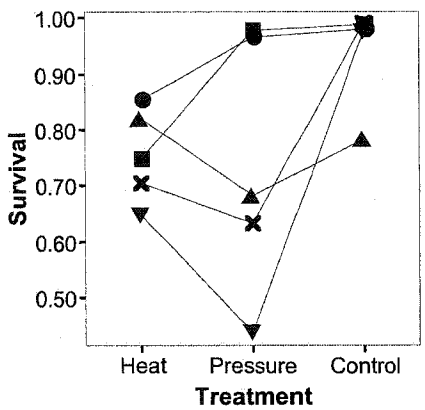
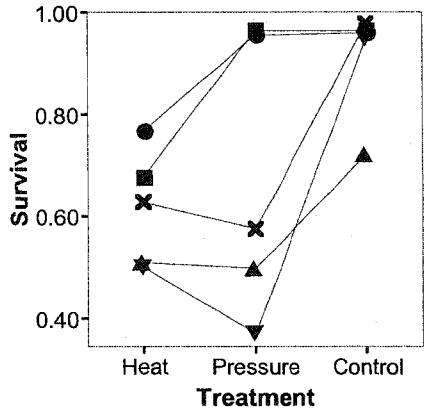


Figure 1.3. Mean cumulative survival (with 95% confidence intervals) of treatment groups through incubation. Survival was calculated as the proportion of surviving embryos/live embryos after mechanical shocking and sorting at the 510, 616, 746, 789, 853 and 970 ATU developmental stages. Circles = 2N-Control, squares = 3N-Pressure, triangles = 3N-Heat.

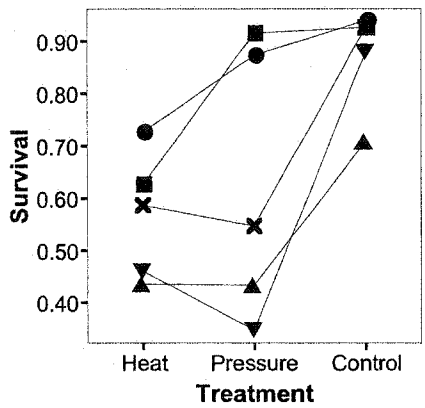
Figure 1.4. Reaction norms for family effects on mean treatment group survival to the 510, 616, 746, 789, 853 and 970 ATU developmental stages during larval incubation (a-f). Survival is calculated as a percentage of live eggs at the eyed stage of development. (actual figure occurs on next page).



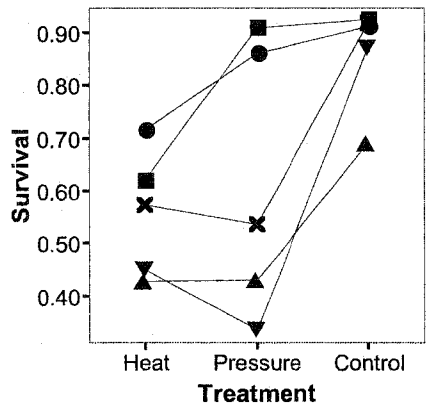
a) 510 ATUs



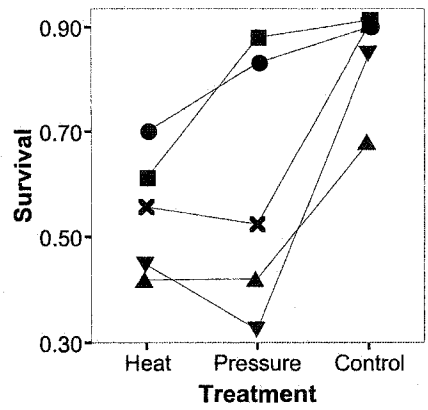
b) 616 ATUs



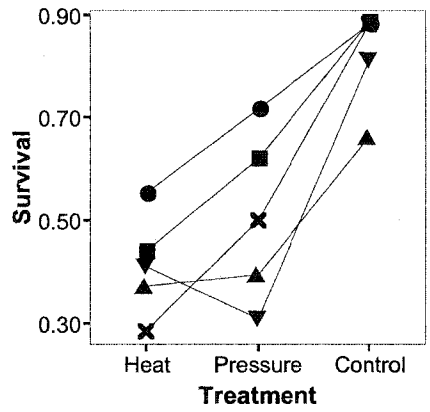
c) 749 ATUs



d) 789 ATUs



e) 853 ATUs



f) 970 ATUs

1.3.3 Survival After Exogenous Feeding

Treatment had a significant and large effect on survival after ponding, as did family ($P < 0.001$ and $P < 0.01$; Table 1.4). While diploid control groups maintained significantly higher mean survival than heat-shock treated groups during this period ($P < 0.001$), control and pressure-shock group mean survival did not differ significantly (mean ± SD; 2N-Control = 0.91 ± 0.07, 3N-Pressure = 0.88 ± 0.08, 3N-Heat = 0.75 ± 0.10; Figure 1.5). Pressure-shock treated fish had significantly higher mean survival than did heat-shock treated fish during this period ($P = 0.003$).The effect of heat-shock treatment on survival through freshwater was clearly negative; heat-shock treatment groups had a mean survival that was 16% below that of control and 13% below that

Table 1.4. Analysis of variance components and omega squared values (ω^2) for freshwater survival after ponding to rearing tanks.

Source of Variation	Sum-of-Squares	df	Mean-Square	F-ratio	ω^2
Treatment	0.294	2	0.147	39.580***	0.42
Family	0.208	4	0.05197	14.015**	0.32
Treatment x Family	0.02967	8	0.003708	0.469	0
Residual	0.119	15	0.0079		

*** Indicates significance at the $P < 0.001$; ** Indicates significance at the $P \leq 0.01$ level

of pressure-shock treatment groups ($P = 0.002$). While there was a significant and large effect of family on survival ($P = 0.001$), it did not appear to be associated with as much of the variation in survival ($\omega^2 = 0.32$) as the effect of treatment ($\omega^2 = 0.42$); there was no significant family by treatment interaction on freshwater survival. There was also no significant effect of rearing tank or the ploidy composition of tank companion groups on survival. Survival was arcsine transformed to attain normality for analysis (Zar 1996); reported means and SDs are non-transformed values.

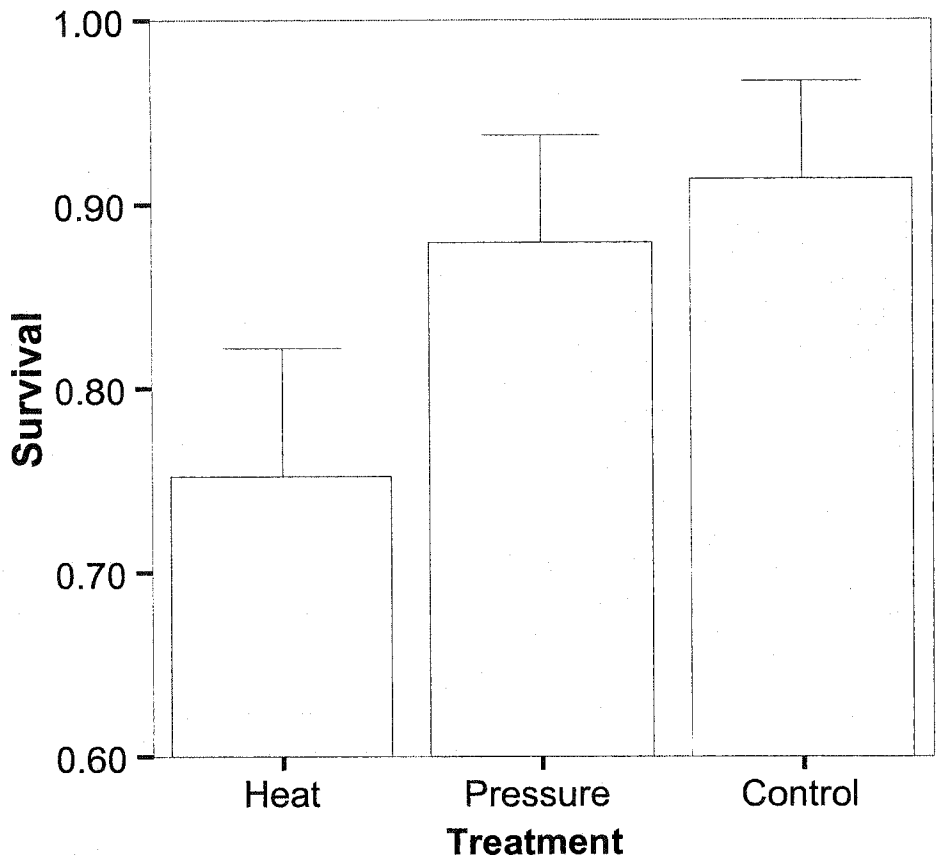


Figure 1.5. Mean survival (95% CI) of treatment groups from the onset of exogenous feeding (ponding) to just prior to saltwater transfer.

1.3.4 Size-at-age.

At ponding, the mean weight of heat- and pressure-shock treated groups were not significantly different from each other ($P = 0.113$) but they were significantly lower than that of the mean weight of diploid control groups (both heat and pressure-shock, $P < 0.001$). Significant effects of family ($P \leq 0.001$) and the interaction between family and treatment ($P \leq 0.001$) on size-at-age existed at each sampled developmental stage after ponding (1224, 1729, 1843 and 1941 ATUs; Table 1.5). Unlike the effect of treatment on

survival, the effect of treatment on size-at-age was not a significant factor at any but the 1729 ATU developmental stage (P = 0.005). The mean weight of diploid control groups

Table 1.5. Analysis of variance components and omega squared values (ω^2) for size-at-age results. Size-at-age was determined at four developmental stages (1224, 1729, 1843 and 1941 ATUs) during freshwater rearing.

Source	Sum-of-Squares	df	Mean-Square	F-ratio	ω^2
1224 ATUs					
Treatment	0.119	2	0.05972	0.919	0
Family	5.858	4	1.465	22.388***	0.61
Treatment x Family	0.528	8	0.06596	10.260***	0.05
Residual	3.626	564	0.006		
1729 ATUs					
Treatment	21.747	2	10.873	11.059**	0.03
Family	98.131	4	24.533	24.851***	0.33
Treatment x Family	7.947	8	0.993	2.625**	0.02
Residual	211.549	559	0.378		
1843 ATUs					
Treatment	32.591	2	16.295	3.569	0.01
Family	298.509	4	74.627	16.353***	0.33
Treatment x Family	36.818	8	4.602	6.541***	0.02
Residual	685.979	975	0.704		
1941 ATUs					
Treatment	32.388	2	16.194	2.963	0.01
Family	343.384	4	85.846	15.525***	0.30
Treatment x Family	44.951	8	5.619	5.705***	0.03
Residual	884.370	898	0.985		

*indicates significance at the P ≤ 0.05 level; **Indicates significance at the P < 0.01level, ***Indicates significance at the P ≤ 0.001 level.

was always significantly higher than the mean weight of triploid groups but the difference in weight was never more than 0.50 grams (1224 ATUS, P = 0.01, heat; P < 0.001, pressure; 1729 ATUs, P < 0.001, heat and pressure; 1843 ATUs, P < 0.001, heat and pressure; 1953 ATUs, P < 0.001, heat and pressure). Heat and pressure shock

treatment group mean weights were never significantly different from each other except at the second freshwater developmental stage (1729 ATUs) when the mean weight of heat-shock treatment groups was 0.15 g heavier than that of pressure-shock groups (Figure 1.6).

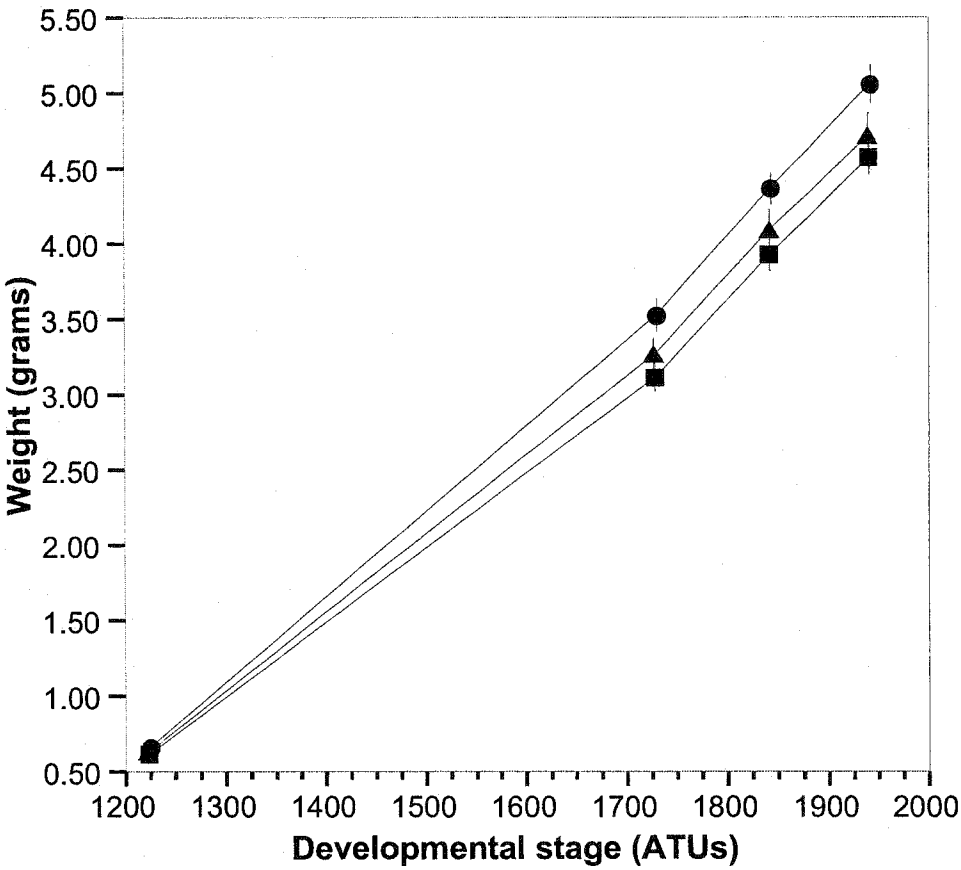


Figure 1.6. Mean size-at-age (weight in grams ± 95% CI) of family treatment groups at three freshwater time points after the onset of exogenous feeding, 1729, 1843 and 1941 ATUs. Triangles = 3N-heat-shock, squares = 3N-pressure-shock, circles = 2N-control.

Reaction norms describing the effect of family on treatment group size-at-age are shown in Figure 1.7. Response to treatment differed significantly across families with an obvious dichotomy establishing early in development between low and high performing

families. Those families that performed poorly as diploids also performed poorly as triploids regardless of treatment and this pattern persisted across the developmental stages at which size-at-age was determined. Surprisingly, the negative response to pressure-shock treatment in size-at-age was most pronounced in families that grew well as diploids and as heat-induced triploids, suggesting that treatment effects may be manifested differently across performance traits; this relationship probably influenced the size of the treatment effect which remained small throughout the freshwater period (as did the interaction effect size) compared to the effect size of family at each stage of development. The effect of family explained 61%, 33%, 33% and 30% of the total variance at each of the size-at-age sample points (Figure 1.7; Table 1.5, refer to ω^2 values). Significant tank effects were found at each developmental stage ($P < 0.05$). To further clarify the nature of tank effects, the effect of the ploidy composition of fish within the tanks on size-at-age was investigated. There was a significant effect of ploidy tank composition on size-at-age at 1224 ATUS ($P = 0.001$), 1789 ATUs ($P < 0.001$), 1853 ATUs (($P < 0.001$) and 1941 ATUs ($P < 0.001$); however tanks composed of two triploidized treatment groups (heat, pressure or mixed heat and pressure) were never significantly different than tanks composed of a mixture of diploid control and triploidized treatment groups (1224 ATUs, $P = 0.507$; 1789 ATUs, $P = 0.944$; 1853 ATUs, $P = 0.554$; 1941 ATUs, $P = 0.175$). Considering that triploidization success rates for heat and pressure treated groups were 97% by flow cytometry (94% and 95% by rbc nuclear analysis), this suggests that rearing fish in low-density mixed ploidy groups did not affect growth. The existence of tank effects even after complete randomization of group placement at ponding may indicate sensitivity of treatment fish to subtle environmental differences inherent in tank position or an interaction between genotype and environment.

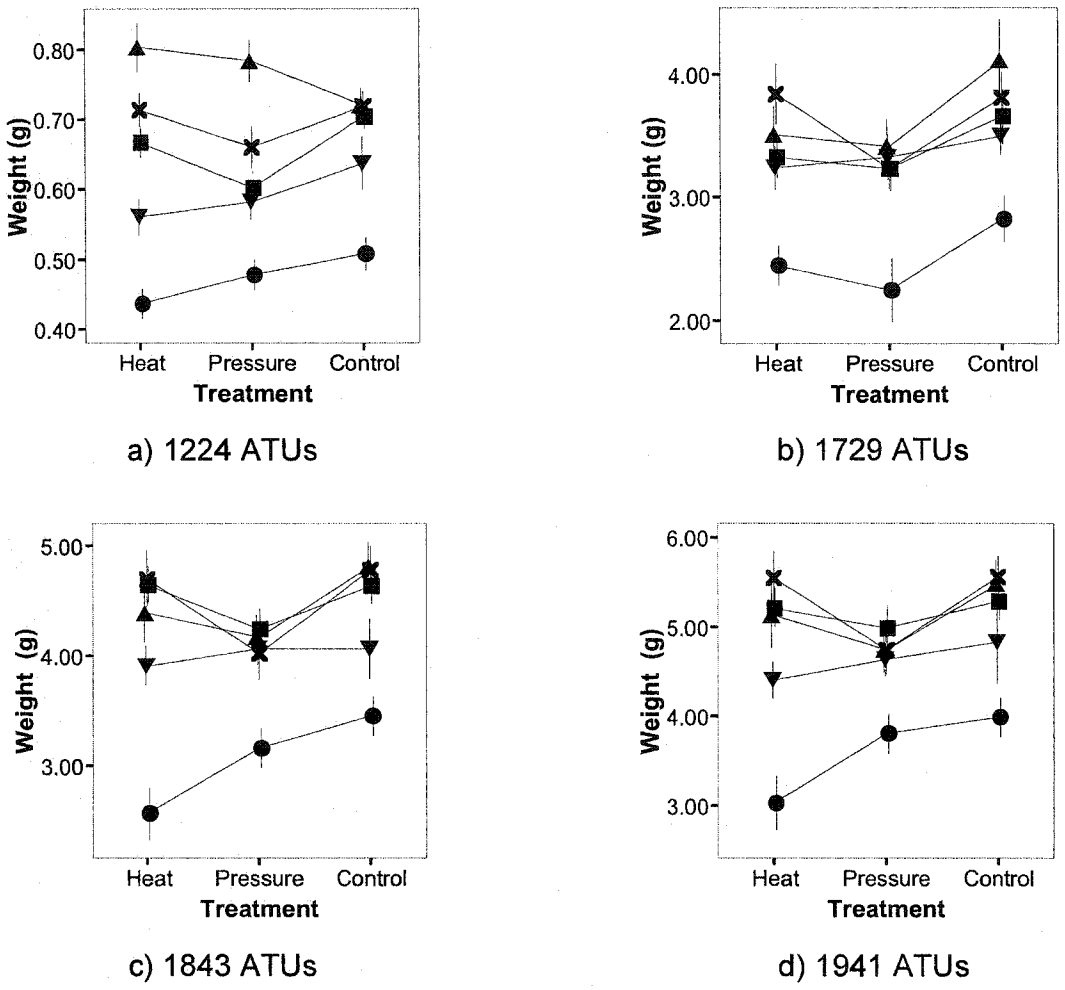


Figure 1.7. Reaction norms for family effects on mean treatment group size-at age through freshwater rearing (95% CI). Graphs a-d depict data for five families at 1224, 1729, 1843 and 1941 ATUs (~ 155, 214, 227 and 236 days post-fertilization) after the onset of exogenous feeding.

1.3.5 Relative Growth Rate.

There were significant effects of family ($P \leq 0.05$) and the interaction between treatment and family ($P \leq 0.001$) on relative growth rate but the effect of treatment was not significant ($P = 0.891$) over the ~100-day period for which freshwater growth was determined (Table 1.6). However, the effect size of the interaction between treatment and family ($\omega^2 = 0.04$) was smaller than the effect size of family (Figure 1.8 and ω^2

values, Table 1.6), which explained 29% of the variation in relative growth rate. Treatment group mean relative growth rates did not differ significantly from each other ($P > 0.05$; 2N-Diploid, mean ± SD = 14.17% ± 3.48%; 3N-Pressure = 14.28% ± 3.41%; 3N-Heat = 14.73% ± 4.06%).

Table 1.6. Analysis of variance components and omega squared values (ω^2) for relative growth rate.

Source of Variation	Sum-of-Squares	df	Mean-Square	F-ratio	ω^2
Treatment	0.005719	2	0.00286	0.117	0
Family	0.490	4	0.123	4.935*	0.29
Treatment x Family	0.202	8	0.02528	10.380***	0.04
Residual	2.187	898	0.001		

*** Indicates significance at the $P \leq 0.001$ level, * $P \leq 0.05$

Reaction norms showing the effect of family on the relative growth rate of treatment groups reflect the pattern exhibited by size-at-age (i.e., low and high performing families) and are plotted in Figure 1.8. Treatment group performance clearly varied with family-of-origin. Families that had low relative growth rates performed poorly regardless of treatment, as did those with relatively high growth rates however, the high performing families appeared to exhibit poor growth when pressure-shock was used for triploidization.

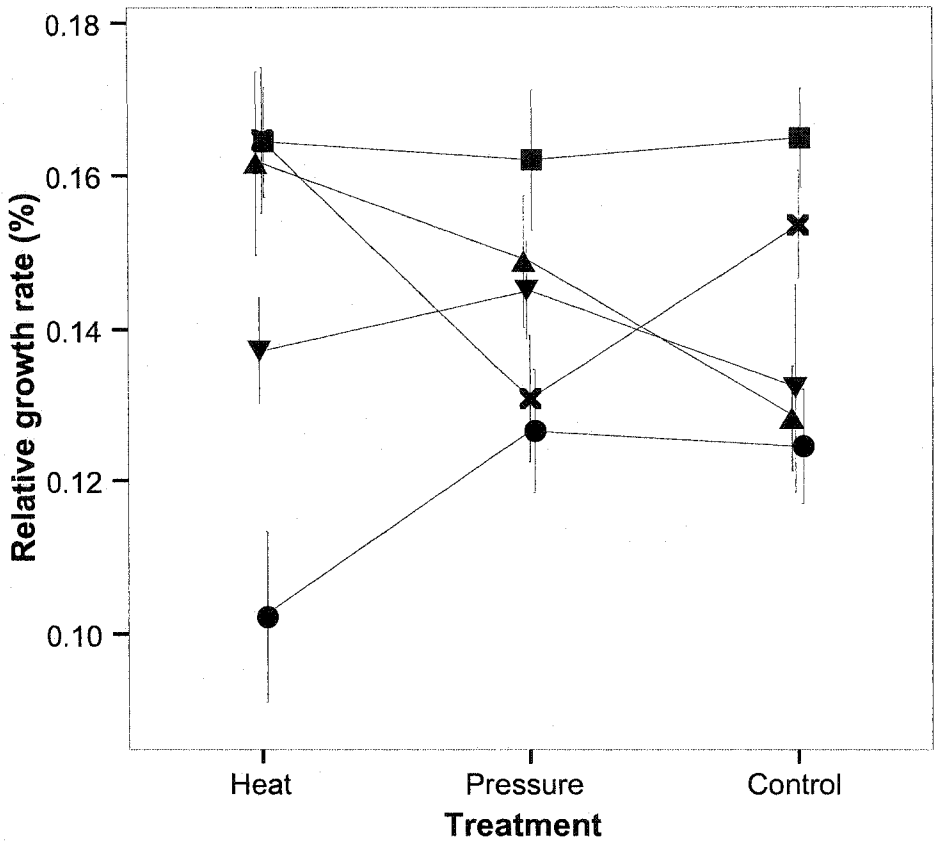


Figure 1.8. Reaction norms (95% CI) for family effects on mean treatment group relative growth rate.

Relative growth rate was arcsine transformed (arcsin$\sqrt{}$) to attain normality for analysis (Zar 1996) but reported means and SDs are non-transformed. There were significant effects of tank ($P < 0.001$) and the ploidy composition of tanks ($P < 0.001$) on relative growth rate. Mixed treatment tanks (heat-shock/diploid-control or pressure-shock/diploid-control) had a significantly lower mean relative growth rate ($P < 0.001$) than did tanks with either 2 diploid groups, 2 heat-shock treated groups or 2 pressure-

shock treated groups ($P < 0.001$) (mean ± SD; all diploid tanks = 15.45% ± 3.29%; triploid treatment tanks = 14.99% ± 3.41%; mixed treatment tanks = 13.67% ± 3.77%)

1.3.6 Response to Vaccination: Enzyme-Linked Immunosorbent Assay

Antibody titers were generally low. Mean serum titration curves for the treatment groups are shown in Figure 1.9. While the mean antibody values for the heat-shocked groups were consistently lower than pressure and control treatment groups over the complete dilution range, the serum dilution of 1/20 was found to be the dilution that best distinguished between mean antibody values of vaccinated (vibrio vaccine) and unvaccinated (sham; PBS) groups. Only a significant effect of the interaction between treatment and family ($P \leq 0.001$) on antibody titer at the 1/20 dilution was found (Table 1.7) but the interaction explained 26% of the total variation in response versus 46% explained by the effect of family (ω^2 values; Table 1.7). The mean OD_{405} values of

Table 1.7. Analysis of variance components and omega squared (ω^2) values for antibody titer. OD_{405nm} values were log transformed for analysis.

Source of Variation	Sum-of-Squares	df	Mean-Square	F-ratio	ω^2
Treatment	0.045	2	0.022	0.680	0.00
Family	0.293	3	0.098	2.979	0.46
Treatment x Family	0.197	6	0.033	5.030***	0.26
Residual	0.235	36	0.007		

*** Indicates significance at the $P < 0.001$ level.

sham-injected treatment groups were significantly different from vaccinated treatment group means but the mean OD_{405} value of vaccinated pressure-shock, heat-shock and diploid control groups were not significantly different from each other (mean OD_{405} ± SD, 3N-Heat = 0.30 ± 0.18, 3N-Pressure = 0.39 ± 0.29, 2N-Control = 0.37 ± 0.29; Figure 1.9). Heat-shock treatment groups appear to vary less between families than do control

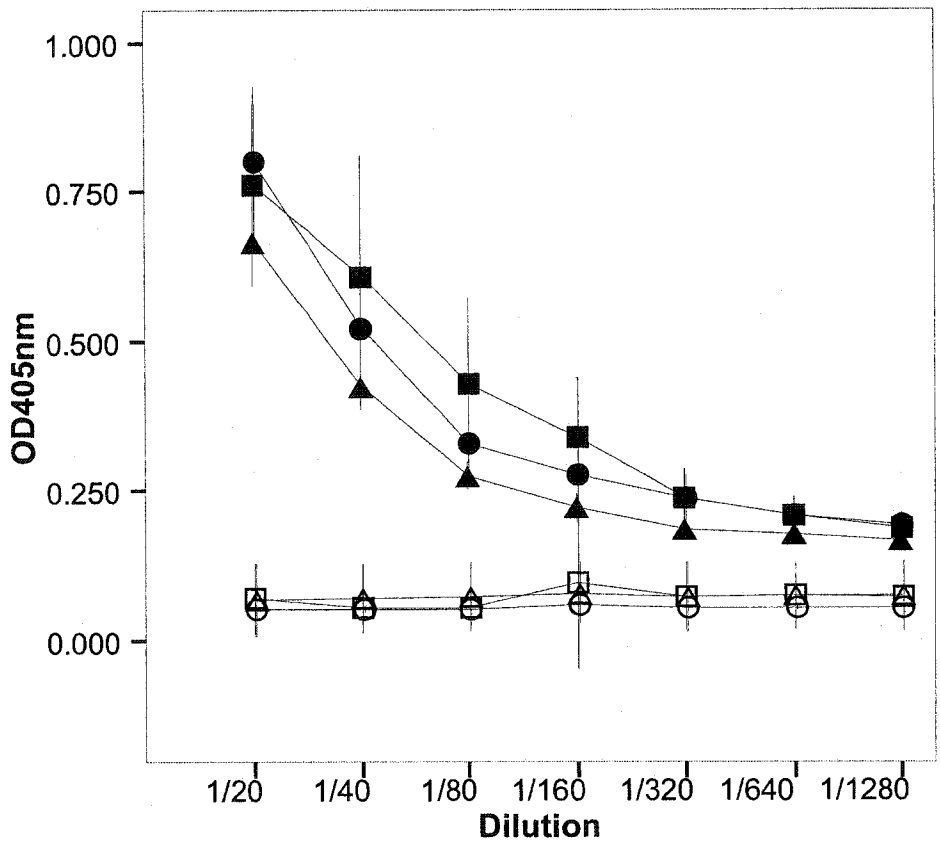


Figure 1.9. ELISA titration curves (95% confidence intervals). Anti-vibrio immune serum titration curves for heat (solid triangles), pressure (solid squares) and control (solid circles) treatment groups as tested by ELISA using *Vibrio anguillarum* as antigen. Lower curves are mean values for titrations of serum from sham-injected treatment groups (PBS); upper curves are mean values for titrations of serum from vaccinated groups (vibrio vaccine). Error bars indicate 95% confidence intervals.

and pressure-shock treatment groups in their response to vaccination but the response is highly variable for all groups (Figure 1.10). Mean weight did not differ significantly between treatment groups ($P = 0.124$; mean ± SD; heat = 3.90 g ± 1.20; pressure = 3.88 g ± 0.94 g; control = 4.48 g ± 0.73 g) and the correlation between OD value andweight was not significant (Pearson correlation = 0.218, $\chi^2 = 2.222, P = 0.14$). OD_{405}

values were log transformed to attain normality for analysis; reported means and SDs are non-transformed values. Red blood cell nuclear analysis confirmed that all heat and pressure shock treated fish tested for an immune response were triploids and all control fish were diploid. There was a significant tank effect on OD_{405nm} ($P < 0.001$) but no significant effect of the ploidy composition of tank (diploid, triploidized, mixed diploid/triploidized) on OD_{405nm}.

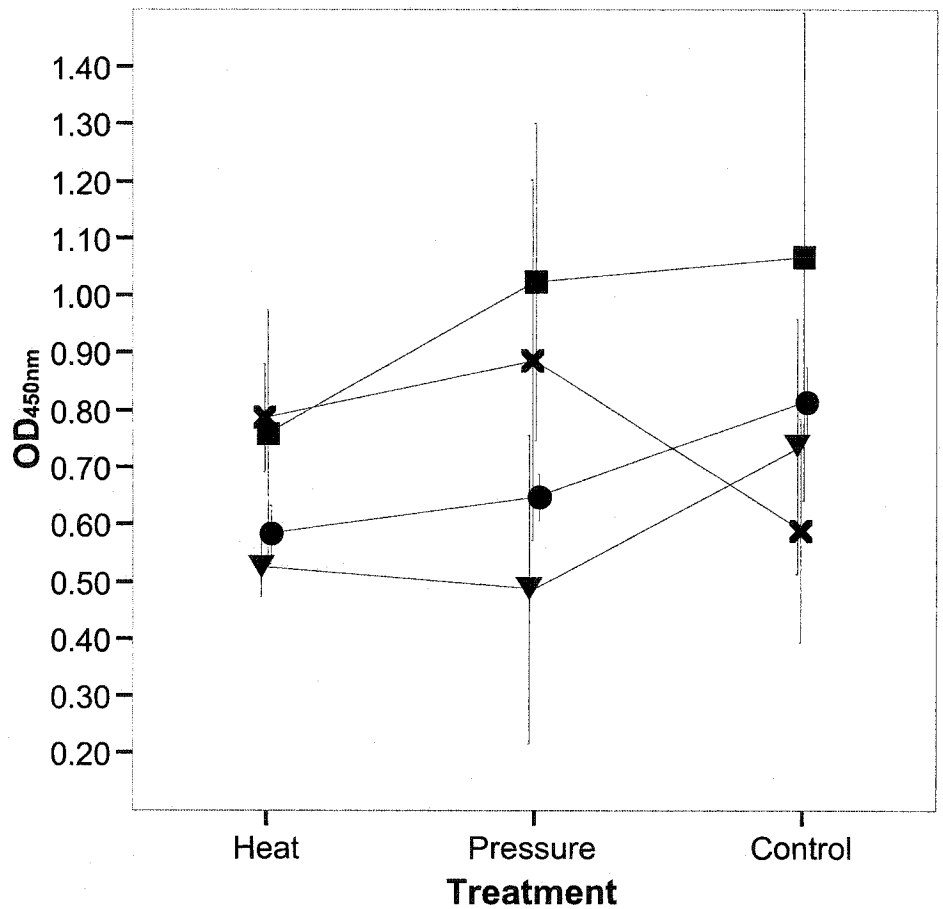


Figure 1.10. Reaction norms (95% CI) for family effects on mean treatment group antibody titre as determined by indirect ELISA. Fish were vaccinated using a commercial vibrio vaccine; *Vibrio anguillarum* was used as antigen in the ELISA.

1.4 Discussion

The first objective of this study was to examine the relative effectiveness of heat and pressure-based induction treatments. Triploidization was most successful when pressure-shock was used for induction, although both methods resulted in high levels of triploidized fish (96% vs 94% triploidization success). However, the slightly lower rate obtained with heat may have occurred due to differences in the consistency of exposure. This may be especially relevant if intra-female egg size is not constant since the surface to volume ratio of eggs will be variable and eggs will differ in the intensity of shock that is received. Interestingly, there was a significant and large interaction between treatment and family that was associated with 53% of the variation in family triploidization success. To my knowledge, this is the first time such an interaction has been reported. Because inter-treatment variability was essentially nil, an interaction of this magnitude is most likely due to genotypic variability in response to treatment potentially caused by inter-female differences in meiotic timing or susceptibility for retention of the polar body (Diaz *et al.* 1993). Differences in triploidization success between females has often been noted (e.g., in newt, coho salmon, and yellow perch Fankhauser and Watson 1942; Habicht *et al.* 1994; Withler, Beacham, Solar, and Donaldson 1995; Withler, Clarke, Blackburn, and Baker 1998; Malison, Procarione, Held, Kayes, and Amundson 1993). It is unlikely that this interaction was due to environmentally determined differences in egg "quality" due to over-ripeness. Maturing females were checked regularly for ovulation and eggs were harvested, fertilized and subjected to triploidization treatments immediately after they were stripped from the females. In this study 31% of the total variation in triploidization success was associated with differences between families (Table 1.1) suggesting the existence of a significant genotypic component.

The second objective of this study was to examine the impact of treatment and family on survival and performance. Triploids are fundamentally different from diploids at a number of different biological levels of organization (i.e., cell size, genetic content). The existence of such radical differences in primary biological structure (Benfey 1999) suggests that integrated physiological functioning might be impaired but that these negative effects might be offset by positive fitness related effects of increased genetic diversity (Leary *et al.* 1985; Allendorf and Leary 1984), reallocation of energy to somatic growth, gene dosage effects (genetic expression directly proportional to ploidy) and/or protection from mutagenic events (Thorgaard *et al.* 1999). Alternatively, disruptions to regulatory and epigenetic pathways (i.e., inherited changes in the patterns of genetic functioning that are not explained by DNA mutation (Russo *et al.* 1996; Spencer 2000; Bird 2002), or inverse dosage effects (i.e., a gene expression activity level that is not directly proportional to gene dosage in a positive manner but rather is reduced as gene copy number increases (Devlin, Holm, Grigliatti 1982; Devlin, Holm, Grigliatti 1988; Birchler, Bhadra, et al 2001) might be compounded with impaired physiological functioning. Additionally, treatment effects caused by the stress of induction might also affect performance of triploids by inflicting structural damage or interfering with the availability or function of embryonic or maternally supplied substances (e.g., mRNA, IgM, proteins) during early development. Because significant interactions were found to exist, main effects were interpreted only when the omega squared effect sizes (ω^2) associated with them were larger than the effect size of the interaction (Sokal and Rohlf 1995).

Treatment was the main factor affecting survival of experimental fish during all stages of development and growth (i.e., to the eyed stage, through the rest of incubation until the start of exogenous feeding, and from the onset of exogenous feeding until the

time of saltwater transfer) and was significantly associated with 34%, 32% and 42% of total variation in survival during the study. However, the effect of family was also large and significant during incubation (after the eyed stage) and after the start of exogenous feeding, explaining 30 % and 32% of the total variation. The interaction effects on survival were significant only during incubation after the eyed stage of development, and accounted for 19% of total variation (versus the 32% and 30% accounted for by treatment and family). The reaction norms showed meaningful interactions throughout incubation, however the magnitude of the interaction decreased by the end of incubation indicating that a shift in the relative importance of factors had occurred. This shift might have been caused by differences in the ability of families to cope with treatment and/or a delayed or threshold embryo response to treatment.

Importantly, there was a relatively consistent ranking of families in terms of survival through incubation and a strong dichotomy between those families performing well as diploids and pressure-induced triploids and those families doing relatively poorly regardless of treatment. High and low performance families were still distinguishable after ponding. This dichotomy suggests that it might be possible to improve survival of triploids using family based selection. However, maternal effects (i.e., the influence of the maternal phenotype and environment on offspring phenotype that is independent of the maternal genetic contribution), cannot be separated from the genetic effects of family using this study design and they are expected to have a large effect on juvenile survival (Heath and Blouw 1998).

In contrast with survival, the effects of treatment and the interaction between treatment and family on growth parameters were minimal while family effects were large (30-61% of the variation in size-at-age and 29% of the variation in relative growth rate). Reaction norms show that the interaction was predominantly due to a pronounced

depression in growth that occurred in three pressure-shock treatment family groups that grew well as heat-shock induced triploids and diploids. This indicates that pressure-shock treatment may affect growth differently from heat-shock. Pressure-shock treatments have been shown to reduce protein synthesis in oocytes and cultured cells (Wilson, Trogadis, Zimmerman, and Zimmerman 2001a; Begg, Salmon, and Hyatt 1983; Symington *et al.* 1991) but extended effects on subsequent juvenile growth seem unlikely unless growth is delayed immediately upon emergence and fish are unable to compensate (i.e., growth rates were depressed in these groups). The large effect of family on weight and growth rate is probably explained by the presence of genotypic effects and partly by the presence of maternal effects that influence offspring size during early development (Heath and Blouw 1998; Berg *et al.* 2001). However, study fish were sampled after the onset of exogenous feeding, approximately 154-258 days post-fertilization when maternal effects are known to be either negative (i.e., offspring tend to resemble paternal phenotypes more than maternal phenotypes) or not significantly different from zero (Heath *et al.* 1999). Ranking of families was relatively consistent throughout freshwater growth and a clear dichotomy between low and high performing families was evident so that those families that grew well as diploids were the ones that also grew well as triploids.

Individual fish serum antibody titre response against *Vibrio anguillarum* antigen varied widely within family treatment groups (but less within heat-shocked family groups) and no significant effect of treatment was detected. These results agree with those of Kusada (1991) who also found no difference in the ability of fish (ayu; *Plecoglossus altivelis*) to respond to vaccination; however agglutination techniques were used in that study. Comparative measurement of diploid-triploid immune system parameters has been limited in salmonids, and most have focused on measurements of

non-specific immunity (Yamamoto and Iida 1995; Kusada, Salati, *et al.* 1991; Benfey 1999). However, the majority of these studies have concluded that diploids and triploids do not differ in their ability to mount effective non-specific immune responses (e.g., haemolytic, bactericidal, neutrophil activity and phagocytosis in rainbow trout and leukocyte profiles in tench, Yamamoto and Iida 1995; Svobodova *et al.* 2001) and are equally responsive to vaccination as measured by mortality after challenge or natural outbreak (e.g., in ayu and African catfish, Inada *et al.* 1990; Na-Nakorn and Lakhaanantakun 1993). However, there is a substantial amount of anecdotal evidence suggesting that triploids are more susceptible to disease than diploids (Ojolick, Cusack, *et al* 1995; Langston, Johnstone, and Ellis 2001; J.W. Heath, personal communication) and recently, differences in the timing and recovery of triploid complement system activity and the hypofaerraemic response to lipopolysaccharide injection were found in Atlantic salmon (Langston *et al.* 2001) suggesting that subtle differences in immune functioning may be discovered by using temporal sampling techniques.

In summary, the full-sib chinook salmon families in this study grew well as triploids and did not exhibit overt differences from diploids in their ability to respond to vaccination. While triploid family groups performed less well than diploids and experienced considerably higher mortality, these differences were either within acceptable bounds for hatchery-reared fish or occurred during embryonic or larval development prior to the onset of exogenous feeding, when selective pressures are intense and the financial investment is low. Overall differences in growth and immune function between heat and pressure-shock treated family groups were found to be minimal indicating that the choice of induction treatment may not be as significant a factor as the quality of diploid broodstock used to generate them. Analysis of overall yield (calculated as the mean replicate group weight x number of surviving fish) prior to

saltwater transfer indicated that the lower survival experienced by heat-shock treated family groups after ponding resulted in a significantly lower yield than was obtained from diploid control groups (P=0.003). Mean yield obtained from pressure-shock treated family groups was not significantly different from that of heat-shock treated groups (P=0.308) or diploid control groups (P = 0.095) indicating that a slight advantage in yield might be gained by using pressure-based induction techniques.

While the industrial application of triploid technology depends on the performance of triploid fish within the commercial environment, it also depends on how well improvements made using the selective breeding of diploids are maintained after triploidization. The obvious dichotomy between high and low performing families evident after triploidization and the large effect of family on survival, growth and the response to vaccination in this study indicates that family selection would be effective. This may be offset somewhat by significant treatment by family interactions and a trend suggesting more variable performance in triploid treatment groups. While this variation might be explained by the increased genetic potential in triploids for either beneficial or detrimental allelic combinations and higher levels of interlocus epistatic interactions, it complicates selection strategies and the ability to predict performance. Identification of the potential sources of this variation (e.g., does triploidization affect the size of the additive genetic component or the magnitude of maternal effects?) is necessary to determine the feasibility of selective breeding programs. While this study design prevented the quantification of additive genetic variation and maternal effects, these issues are addressed in the study included in Chapter Two of this thesis.

Chapter 2

QUANTITATIVE GENETIC ANALYSIS OF DIPLOID AND TRIPLOID CHINOOK SALMON PERFORMANCE CHARACTERISTICS

2.0 Abstract

Monosex all-female chinook salmon families bred using a paternal half-sib breeding design (62 females and 31 males) were used to test whether triploidization resulted in changes in: 1) the distribution and magnitude of phenotypic variation, 2) narrow-sense heritability and 3) maternal effects, of specific fitness-related parameters (i.e., of survival, size-at-age, relative growth rate and serum lysozyme activity) measured during the freshwater phase of the lifecycle. Analysis was performed separately for diploid and triploid family groups. It was found that triploidization resulted in significantly higher levels of phenotypic variance and profoundly different patterns of variance distribution, although this relationship was reversed for lysozyme activity. Additive genetic variance accounted for much more of the total phenotypic variance in triploids and this resulted in significantly higher narrow sense heritability values for triploid groups. However, maternal effects estimates were substantially lower in triploids than in diploids. These results indicate that the main effects of adding an extra set of chromosomes to the chinook salmon genome are primarily additive and dominant and that, somewhat counter-intuitively, the relative magnitude of the combined effect of dominance, epistasis and maternal effects is not increased. This is highly suggestive of an overall ploidy dependent mode of gene expression.

2.1 Introduction

Successful triploidization results in a balanced or euploid chromosomal state because an entire set of chromosomes is retained by the zygote. In triploid chinook salmon this means that the chromosome number increases from 68 (the diploid number) to 102 chromosomes (Simon 1963; Phillips and Rab 2001). Unlike aneuploidy, in which a single chromosome or gene construct is added to the genome, triploidy does not always result in potentially catastrophic genomic imbalance. However, triploidy does increase bulk DNA content, the number of alleles at each locus and, potentially, the interactions among loci. These fundamental changes may modify relationships within (dominance) and between (epistasis) loci, with resultant alterations in gene expression and ultimately phenotype.

Gene expression may be altered in a number of different ways including regulatory factor effects, RNA mediated interference and homology dependent recognition and silencing (Wassenegger 2002a; Wassenegger 2002b). Modulation of gene expression may result in gene dosage effects or dosage compensation. When a dosage effect occurs, gene expression is correlated with the number of copies of the structural gene (ploidy in the case of euploids). For example, haploids, diploids and triploids would have gene expression levels of 50%, 100% and 150% (positive gene dosage effect) or 200%, 100% and 67% (inverse gene dosage effect) (Birchler *et al.* 2001). Dosage compensation may also occur whereby a positive gene dosage effect is compensated by an inverse effect of another regulatory product on the structural gene leading to gene expression at diploid levels regardless of genomic ploidy state (Birchler *et al* 2001).

The addition of a complete set of chromosomes probably does not disrupt gene expression patterns to the same degree as genomic manipulations that generate

aneuploidies because *overall* stoichiometric relationships are not disrupted and the cytoplasmic:nuclear ratio is generally preserved, so that the concentration of regulatory factors is likely maintained (Birchler *et al.* 2001). However, this has not been investigated in a vertebrate ploidy series (i.e, groups of organisms in which the number of complete chromosome sets is varied sequentially). Evidence from plant and non-vertebrate ploidy series generated using corn (*Zea mays*), fruitfly (*Drosophila melanogaster)* and yeast (*Saccharomyces cerevisiae)* have found that gene expression in polyploids tends to be positively associated with ploidy so that expression of specific genes increases in a linear manner as ploidy is increased (i.e., a positive gene dosage effect is exhibited) (Guo, Davis, and Birchler 1996; Lucchesi and Rawls 1973; Birchler *et al.* 1990; Galitski *et al.* 1999). Although some genes were found to have unusually high or low expression patterns outside of the range of simple gene dosage or dosage compensation effects (e.g., Guo *et al.* 1996), positive gene dosage effects appear to be the most prevalent form of modified expression in ploidy series experiments. However, a recent study using a silkworm (*Bombyx mori*) ploidy series suggests that a more complex relationship exists between ploidy state, parental origin of chromosome sets and parental specific regulatory factor influences on expression (Suzuki *et al.* 1999).

Triploidization can be induced when a shock (typically heat, pressure or chemical) is applied to a fertilized egg just prior to second polar body extrusion. This shock, if applied successfully, causes the set of chromosomes within the polar body to be retained within the egg. Shock-induced triploidization is a stressful and highly perturbational event. Salmon zygotes subjected to induction must cope with potential treatment-related trauma as well as possible developmental, cellular, regulatory and phenotypic perturbations related to the forced transformation of genetic background caused by retention of an extra set of chromosomes. Despite this, typical cellular-level

compensatory responses to triploidization are displayed by salmonids (e.g., increased nuclear and cell size with apparent maintenance of the diploid nuclear to cytoplasmic ratio) and physiological parameters are remarkably similar to those of diploids (reviewed by Benfey 1999).

Genetic changes associated with shock-induced triploidy in salmonids have not been thoroughly investigated. However, it is known that in addition to the increase in DNA quantity, shock-induced triploidy increases allelic diversity and number (i.e. three versus two alleles at each locus and potentially an additional *different* allele per locus (depending on recombination rate) (Thorgaard *et al.* 1983; Allendorf and Leary 1984; Leary *et al.* 1985). This increased genetic diversity might be expected to have positive fitness-related effects (e.g., deleterious alleles may have a higher probability of being masked or a synergistically favorable combination of alleles may occur (Garnier-Gere *et al.* 2002; Wang *et al.* 2002). However, the increased structural complexity implicit in triploid genomic architecture may alter allele and gene interactions (i.e., dominance and epistasis), alter specific regulatory factor stoichiometry, epigenetic, or developmental gene regulation so that gene expression patterns might be affected in a detrimental or stochastic manner. Furthermore, since genotype and phenotype are fundamentally linked through the patterns of gene expression during development, changes in genetic architecture (e.g., modifications of epistatic relationships among sets of developmentally important genes) caused by triploidization may modify expression by changing regulatory control of transcription patterns during development, and thus modify phenotypic potential.

It is unknown if gene expression patterns change after triploidization or if epigenetic regulation occurs in salmon species. Gene expression studies of triploid salmon should be able to quantify the detailed effect of triploidization on expression

patterns of a specific sub-set of genes and clarify the role of parental or strain specific regulatory factor influences on gene expression. Quantitative genetic analysis on the other hand, would allow a direct estimation of the average phenotypic change (over all alleles at contributing loci) in additive genetic variance attributable to ploidy modification. This analysis would specifically entail the decomposition of phenotypic variance and comparative diploid/triploid estimates of narrow sense heritability (h^2, the additive genetic component of the phenotypic variance of a trait). If complete or partial dosage compensation is occurring in triploids, then heritability values might be expected to be similar to those of diploids; however, if there is incomplete dosage compensation then heritability values might be expected to be significantly larger in triploids. Results obtained in Chapter One and recent published work (Bonnet *et al.* 1999; Blanc *et al.* 2001 and Friars *et al.* 2001) suggest that phenotypic variance is increased in triploid salmon. If all or most gene action is additive there should be a linear relationship between phenotypic variance, allelic or genetic diversity and additive genetic variation (Falconer and Mackay 1996; Reed and Frankham 2001). Such a relationship would predict higher triploid phenotypic variance and heritability values relative to diploids.

Triploidization may also modify dominance related interactions between alleles (*the phenotypic effect of the interaction of alleles at single loci*, Falconer and Mackay 1996) as well as epistatic interactions among loci (the phenotypic effect of gene interactions, Cheverud and Routman 1995). Epistasis and dominance may contribute to additive genetic variance and inflate heritability estimates under certain conditions and allele frequencies (e.g., perturbation of genetic background, population bottlenecks) (Willis and Orr 1993; Whitlock *et al.* 1993; Cheverud and Routman 1995; Lynch and Walsh 1998). In quantitative genetic analyses, epistatic variance components are usually considered negligible and so are generally ignored, and if main effects are

primarily additive or dominant then this may be valid (Falconer and Mackay 1996; Roff 1997; Wade 2002).

In the present study, a suite of monosex all-female chinook salmon families (*Oncorhynchus tshawytscha*) bred using a paternal half-sib mating design was used to test whether triploidization resulted in changes in: 1) the distribution or magnitude of phenotypic variation, 2) narrow-sense heritability and 3) maternal effects. Maternal effects occur when the phenotype or genotype of the mother, or the environment she experiences has a phenotypic effect on her offspring (Rossiter 1996; Mousseau and Fox 1998; McAdam *et al.* 2002). Although this analysis is primarily designed to test for changes in the nature of quantitative trait expression in diploid and triploid salmon, the results will have relevance for aquaculture as well. The potential for significant changes in the inheritance patterns of performance traits in triploid offspring from a high-performance broodstock has serious implications for the application of triploid sterilization in commercial salmon aquaculture.

2.2 Methods

2.2.1 Fish, Breeding Design & Treatment Details

Monosex all-female chinook salmon broodstock from Yellow Island Aquaculture Ltd. (YIAL; Quadra Island, B.C.) were mated using a paternal half-sib design. In this mating scheme, each of 31 hormonally masculinized phenotypic males (neomales) were mated to two independent, non-related and randomly chosen females (females were bred once and only to one male). The breeding design resulted in a total of 62 full-sib families nested within 31 paternal half-sib groups. The fertilized eggs from each full-sib family were divided into two 250 ml sub-samples, each of which was subjected to one of the following two treatments: i) *Hydrostatic pressure-shock.* 6.89 x 10^4 kPa (10

000 psi) of pressure applied for 5 minutes, 30 minutes after fertilization or, ii) *Control.* Eggs were left untreated and transferred to incubation trays immediately after the water hardening process.

2.2.2. Fish Rearing & Husbandry

Eggs from each family treatment group were incubated in separate compartments of vertical stack incubation trays (Heath Techna Corp.). When eggs reached the eyed stage of development (the point at which the eye spots of the developing embryo are visible through the egg shell and at which ¾ yolk vascularization has occurred; November 28-December 11, 2000, ~280-296 ATUs) they were mechanically shocked and sorted using a Jensorter machine (Model JM4C, Jensorter, Inc.) and returned to the incubation stacks. Trays were divided into twelve compartments (10 cm x 10 cm x 5cm) with each compartment holding approximately 700 eggs. Water temperature within the stacks was monitored using a digital data logger (Onset Computer Corp.) and development stage of the fish was tracked using Accumulated Temperature Units (ATUs; calculated as the cumulative total of daily mean temperatures). Mean water temperature during the incubation period was 7.72 °C ± 0.02 °C. Mean flow within the stacks was 13 L/minute.

As alevins completed yolk-sac absorbtion (February 24-March 11, 2001; ~927-1006 ATUs), 100 alevins were randomly selected from the treatment groups (pressure and control) within each of the 62 full-sib families and transferred from the vertical incubation stack tray compartments to 140 L aerated rearing tanks for the onset of exogenous feeding. Two sets of fish were randomly assigned to each tank at a starting density of 200 fish per tank (~1.43 fish/L). One set of fish in each tank was fin clipped for identification purposes (either the upper or lower caudal fin lobe was removed).

Flow rate of water to the tanks was approximately 3 L/minute and the mean temperature (February 24th-July 1) was 8.79 °C (7.23 -10.18 °C). Fish were handfed to satiation multiple times per day with commercial feed (Ewos, Canada, Ltd.).

2.2.3. Ploidy Determination

Erythrocyte nuclear length was used to determine family-specific and overall triploidization success (Wolters *et al.* 1982; Beck and Biggers 1983; Benfey *et al.* 1984). The validity of this measurement had been specifically tested against flow cytometric data during preliminary analysis of juvenile chinook at YIAL and was confirmed again for the analysis of chapter one data. A nuclear length of 8.5 μm was found to be the threshold measurement that most reliably distinguished diploid from triploid individuals. Approximately 11-20 fish were terminally sampled from each family treatment group (pressure = 1084 fish; control = 997) and whole blood smears were made for each fish. The length of ten randomly chosen erythrocyte nuclei per smear was measured to the nearest 0.01μm and the mean nuclear length used to determine the ploidy status of each fish. Slides were fixed in methanol and stained with Wright-Giemsa (Sigma). Visualization and measurement of erythrocyte nuclei was accomplished under oil immersion (1000x magnification) using an Olympus BX-50 compound microscope (Olympus Optical Co.) equipped with a QImaging Retiga 1300 Monochromatic digital camera (Quantitative Imaging Corp.) and the Northern Eclipse, version 6.0 imaging program (Empix Imaging Inc.).

2.2.4 Survival

Incubation survival of family treatment groups was monitored from fertilization to the eyed stage of development (S-1) and then followed through to the alevin stage, just

prior to transfer of the fish to freshwater rearing tanks. Embryo mortalities from fertilization to the eyed stage (3/4 yolk vascularization) were assessed after the eggs were mechanically shocked and sorted using a Jensorter machine. Total egg number and the number of live eggs for each family treatment group were evaluated at this time. After the initial eyed egg count, mortality was monitored at least every two days. Incubation survival was determined for all groups at the following developmental stages, S-2, S-3, S-4, S-5, S-6, S-7. Refer to Table 2.1 below, for a summary of the performance variables measured in this study and the corresponding in text abbreviations.

Survival after transfer to rearing tanks (S-8) was also monitored every second day and was determined as the number of live fish remaining immediately prior to the experimental vaccination treatment divided by the total number of fish originally fin-clipped and released into the rearing tank. Experimentally sacrificed fish within each group (i.e., those fish terminally sampled to determine triploidization success) were excluded from the final survival assessment.

2.2.5 Growth

Weight (in grams) was determined by non-terminal sampling at five time points during the freshwater growth of the ponded family groups: W-0, W-1, W-2, W-3 and W-4 (Refer to Table 2.1 for specific definitions). At ponding (W-0), 100 alevins from each family treatment group were weighed in water as they were transferred to rearing tanks so that a mean family weight was obtained at this sample point. At all other sample times, weights of individual fish were recorded. Approximately twenty fish per family treatment group (pressure, control) were weighed at the W-1 (n = 2460) and W-2 (n = 2568) sample points. At the W-3 (n = 1290) and W-4 (n = 1157) sample points, 40 fish

per family treatment group were weighed.

Table 2.1. Summary of measured performance variables and in-text abbreviations

Abbreviation	Performance Variable
Incubation survival (stage-specific)	
S-1	*Fert-288 ATUs (eyed stage)*
S-2	*~288- 510 ATUs*
S-3	*~510- 616 ATUs*
S-4	*~616- 746 ATUs*
S-5	*~746- 789 ATUs*
S-6	*~789- 853 ATUs*
S-7	*~853-970 ATUs*
Survival after ponding to rearing tanks	
S-8	*~970-2008 ATUs (~127-244 days post-fert)*
Size-at-age (weight, grams)	
W-0	*~970 ATUs (~127 days post-fert)**
W-1	*~1228 ATUs (~159 days post-fert)*
W-2	*~1729 ATUs (~215 days post-fert)*
W-3	*~1835 ATUs (~235 days post-fert)*
W-4	*~1943 ATUs (~244 days post-fert)*
Relative growth rate	
rgr-c	*~970-1943 ATUs (~127-244 days post-fert)*
rgr-1	*~ 970-1228 ATUs (~127-159 days post-fert)*
rgr-2	*~1228-1729 ATUs (~159-215 days post-fert)*
rgr-3	*~1729-1943 ATUs (~215-244 days post-fert)*
Serum lysozyme activity (EU/5 µl)	
SLR	*Pre-post-vaccination difference*

* **NOTE:** W-0 = the mean weight of 100 alevins per family treatment group (weighed in water at the time of ponding)

Relative growth rate of replicate family treatment groups was assessed for the complete period of growth after ponding, a time interval of ~100 days (rgr-c in Table 2.1) but was also calculated for specific sub-periods: rgr-1 (32 days), rgr-2 (56 days) and rgr-3 (29 days) in Table 2.1. Because individual fish were not tracked, mean replicate treatment group weight data were used for the first sampling point and individual fish weights were used for the second sampling point. Relative growth rate was calculated using the following formula:

$$\left\{\left(Y_2 - \overline{Y_1}\right) \div \overline{Y_1}\left(t_2 - t_1\right)\right\} \times 100$$

where, Y_2 = individual fish weight at the second sampling point, $\overline{Y_1}$ = mean treatment group weight at the first sampling point and $t_2 - t_1$ = the mean time interval in days between the first and second sampling points.

2.2.6. Vaccination & Serum Lysozyme Activity Assay

To determine if pre- and post-vaccination serum lysozyme activity levels differed between ploidy types, approximately 15 fish per family treatment group were terminally sampled just prior to (June 6-14th, 2001) and 10 days after vaccination (June 24-25th) with a commercial vibrio vaccine (Alpha-Dip 2100, *Vibrio anguillarum*, serotype 01 and *V. ordalii* bacterin; Alpharma NW Inc.). Vaccine was diluted 1:9 with hatchery water and fish were immersed for 30 seconds in the aerated solution before being returned to rearing tanks (vaccination occurred June 14th-15th). Pre-immunization serum lysozyme activity levels were determined for 769 fish (2N-Control = 379, 3N-Pressure = 390) and post-immunization serum lysozyme activity levels were determined for 842 fish (2N-Control = 390, 3N-Pressure = 452) using the modified microplate assay protocol of Rungruangsak -Torrissen (Rungruangsak -Torrissen *et al.* 1999) based on Ellis (1993).

Briefly, 5 µl of undiluted serum were placed into wells of a 96-well microplate; 95 µl of a 0.21 mg/mL *Micrococcus lysodeikticus* –0.05 M phosphate buffered saline solution was then added quickly to all wells using a multi-channel pipettor and the absorbance at 450nm was measured after 1 and 5 minutes at 25 °C using a VERSAmax tunable plate reader with Softmax Pro 4.0 software (Molecular Devices, Corp.). Thirty-two serum samples were run in duplicate on each plate with two columns of wells used to run a series of hen egg white controls (4-1000 µg/mL HEWL diluted in 0.05 M PBS), one column of wells was left as a series of blank controls, and one column of wells was run as internal PBS controls. Serum samples with absorbance values outside the range of the HEWL controls were run again in dilution. The enzymatic activity of the HEWL standards was determined using a quality control assay. One unit of lysozyme activity (EU) was defined as the amount of enzyme causing a decrease in A_{450nm} of 0.001/minute. The response to vaccination was determined as the individual fish post-vaccination enzyme activity level minus the mean family pre-vaccination enzyme activity level. Sample activity levels were expressed as enzyme units/mL (EU/mL) and were $\log_{10}$ transformed to attain normality for statistical analysis.

2.2.7 Statistical Analyses

One-way analysis of variance (ANOVA) was used to determine the effect of treatment on measured performance parameters. One-way ANOVA was again used to determine how total phenotypic variance of each performance trait was partitioned within and among families. Pitman's procedure for correlated populations was used to test for significant differences between variances (Zar 1996). Analysis was run separately for each treatment group/performance trait combination.

To partition phenotypic variance for the quantitative genetic analysis, nested

ANOVA was used with sire and dam factors treated as random effects (Model II). Variance was partitioned between sires, between dams nested within sire, [dam(sire)], and between offspring nested within dam, [offspring(dam)]) using the following linear model:

$$Y_{ijk} = \mu + A_i + B_{ij} + \varepsilon_{ijk}$$

where, Y_{ijk} is the phenotype of the kth offspring from the family of the ith sire mated to the jth dam, μ is the parametric mean of the population, A_i is the random effect of the ith sire, B_{ij} is the random effect of the jth dam mated to the ith sire and ε_{ijk} is the residual deviation (Sokal and Rohlf 1995).

Sire and dam additive genetic components (heritabilities, h^2_{sire}, h^2_{dam}) were estimated using the appropriate mean squares from the nested ANOVAs and variance components calculated using standard formulas as outlined in Roff (1997) and Falconer and Mackay (1996). Standard errors of sire and dam heritabilities were estimated using the appropriate intraclass correlation coefficients using the techniques of Robertson (1959). The sire heritability was used as the best estimate of the additive genetic component because it is not inflated by variance due to dominance or maternal effects and probably only minimally inflated by epistatic effects (Roff 1997), at least for diploids. Sire heritabilities were considered significantly different from zero when the F-value derived from the analysis of variance indicated a significant sire effect regardless of whether the 95% confidence interval of the h^2 estimate (derived as 1.96 x SE of the h^2 value) encompassed zero (Roff 1997). It is important to recognize that an assumption of negligible epistatic effects has been made in the analysis. This may not be a valid assumption for the calculation of heritability for triploids where the probability for epistatic interactions is increased. But, as Roff (1997) states, epistatic interactions will

tend to inflate the additive and dominance components of variance and so would probably appear as inflated sire or dam heritability components.

The paternal half-sib model employed in this study does not allow for the specific decomposition of epistatic, maternal effects or dominance variances. Despite this, a general estimate of the magnitude of maternal effects was calculated using the difference between the dam and sire causal components of variance divided by the total phenotypic variance for each performance trait. The dam component contains all variance due to maternal effects (genetic and non-genetic), one quarter of the variance attributable to dominance and small proportions of epistatic variance (i.e., 3/16 additive x additive, 1/8 additive x dominance and 1/16 dominance x dominance). If dominance and/or epistasis is present (or inflated by triploidization) it will be confounded with the maternal effects estimate (Roff 1997). The study design thus allows maternal effects to be detected and generally estimated, but the maternal effects estimate cannot be decomposed and the presence of dominance and/or epistasis may inflate the estimate (Roff 1997). Analysis was performed separately for diploid control and triploid pressure-shock treatment groups for each trait. Differences between diploid and triploid heritabiity estimates were examined and a paired sample nonparametric sign test was used to identify the probability of obtaining the observed distribution of differences (Zar 1996).

Sire and dam heritabilities were estimated for triploidization success (a threshold trait) in pressure-shock treated fish first on a 0, 1 scale (where, 1 = triploid and 0 = diploid) according to Roff (1997) and then on the underlying or liability (Falconer and Mackay 1996) scale according to Dempster and Lerner (1950) using Hamaker's (1978) exact approximation of the *z* value. Standard errors and maternal effects estimates were calculated as above.

2.3 Results

2.3.1 Patterns of Variance Distribution Among and Within Families

Triploidization resulted in increased overall phenotypic variance (Table 2.2). Triploids exhibited significantly higher ($P < 0.05$) phenotypic variance among families than diploids for most traits (i.e., for survival, both during incubation and after ponding, size-at-age and relative growth rate) with the exception of serum lysozyme activity. However, diploids did have significantly higher among family variance for incubation survival over the fourth and last sample periods, S-4 and S-7, and for relative growth rate during the last sample period, rgr-3. Within family variance of treatment group size-at-age was not significantly different between ploidy types at any of the post-fertilization sample points (W-1 to W-4) but triploids had significantly higher within family variance for the first and third sample periods (rgr-1, rgr-3 and rgr-c). Diploid within family variance was significantly higher for relative growth rate over the complete study period (rgr-c). Interestingly, the trend indicating increased variance among and within triploid families was reversed for the difference between pre- and post vaccination serum lysozyme activity. Diploids exhibited significantly higher among ($P < 0.05$) and within family variance ($P < 0.001$) than did triploids.

2.3.2 Sib analysis, Narrow Sense Heritability and Maternal Effects

A number of interesting differences between diploids and triploids were noted in the partitioning of phenotypic variance among sires, among dams nested within sires,and among progeny within dams during the sib analysis (Table 2.3 and 2.4) and estimation of heritability (Table 2.5). Results are reported in terms of the distribution of variance, the relative contribution of causal components along with the heritability and maternal effects estimates (Table 2.5) separately for each performance trait below.

Table 2.2. Among and within family phenotypic variance of measured traits in diploid control and triploid pressure-shock treated family groups. *, $P \leq 0.05$; ** $P \leq 0.01$, ***, $P \leq 0.001$. Trait abbreviations are as defined in Table 2.1. N/A = not applicable.

Trait	Among Family Phenotypic Variance		Within Family Phenotypic Variance	
	2N - Control	**3N - Pressure**	**2N - Control**	**3N - Pressure**
Incubation survival (stage-specific)				
S-1	3.337×10^{-2}	$5.521 \times 10^{-2*}$	N/A	N/A
S-2	1.343×10^{-2}	$4.624 \times 10^{-2***}$	N/A	N/A
S-3	3.855×10^{-2}	$5.781 \times 10^{-2**}$	N/A	N/A
S-4	7.092×10^{-2}	$5.253 \times 10^{-2*}$	N/A	N/A
S-5	3.691×10^{-2}	$4.827 \times 10^{-2**}$	N/A	N/A
S-6	3.795×10^{-2}	$5.218 \times 10^{-2**}$	N/A	N/A
S-7	$7.889 \times 10^{-2*}$	7.542×10^{-2}	N/A	N/A
Survival after ponding to rearing tanks				
S-8	3.434×10^{-2}	$6.293 \times 10^{-2**}$	N/A	N/A
Size-at-age (weight, grams)				
W-1	6.794×10^{-3}	$1.011 \times 10^{-2*}$	9.761×10^{-3}	1.101×10^{-2}
W-2	1.206×10^{-1}	$2.010 \times 10^{-1*}$	0.436	0.457
W-3	1.073×10^{-2}	$1.597 \times 10^{-1*}$	0.605	0.526
W-4	1.847×10^{-1}	$2.834 \times 10^{-1*}$	1.201	1.152
Relative growth rate				
Rgr-c	7.989×10^{-5}	$1.031 \times 10^{-4*}$	$6.501 \times 10^{-4*}$	6.388×10^{-4}
Rgr-1	1.830×10^{-5}	$2.135 \times 10^{-5*}$	4.596×10^{-5}	$5.092 \times 10^{-5*}$
Rgr-2	5.323×10^{-5}	$9.443 \times 10^{-5*}$	$2.354 \times 10^{-4*}$	2.228×10^{-4}
Rgr-3	$5.483 \times 10^{-6*}$	4.925×10^{-6}	9.767×10^{-5}	$9.872 \times 10^{-5*}$
Serum lysozyme activity (EU/5 µl)				
SLR	0.9868*	0.8646	13.954***	3.205

Table 2.3. Results of nested Model II ANOVA and calculated variance components for the heritability analysis of size-at-age and serum lysozyme activity measured in diploid control and triploid pressure-shock family treatment groups. *, $P \leq 0.05$; **, $P \leq 0.01$; *** $P \leq 0.001$.

Trait	Source	Diploid Control				Triploid Pressure-Shock			
		SS	*df*	*MS*	*Var.*	*SS*	*df*	*MS*	*Var.*
Size-at-age									
W-1	Sire	15.046	29	0.519	0.004	28.455	30	0.948	0.0116*
	Dam(sire)	10.841	30	0.361	0.018***	15.063	31	0.486	0.0238***
	Offspring	11.111	1142	0.010	0.010	12.137	1172	0.010	0.0100
W-2	Sire	198.968	29	6.861	-0.065	377.653	30	12.588	-0.039
	Dam(sire)	284.379	30	9.479	0.452***	439.169	31	14.167	0.686***
	Offspring	494.618	1139	0.434	0.434	535.803	1175	0.456	0.456
W-3	Sire	192.417	28	6.872	-0.005	338.050	28	12.073	0.251
	Dam(sire)	201.986	29	6.965	0.634***	204.546	29	7.053	0.654***
	Offspring	324.637	523	0.621	0.621	269.725	521	0.518	0.518
W-4	Sire	371.881	28	13.281	0.103*	696.174	28	24.863	0.565*
	Dam(sire)	325.343	29	11.219	0.997***	393.470	29	13.568	1.255***
	Offspring	650.349	522	1.246	1.246	522.665	515	1.015	1.015
Lysozyme activity									
SLR	Sire	1162.682	17	68.393	-4.048	1738.701	21	82.795	0.490
	Dam(sire)	2178.403	18	121.022	12.966***	1670.575	22	75.935	8.464***
	Offspring	6022.107	199	30.262	30.262	4222.691	253	16.690	16.690

Table 2.4. Results of nested Model II ANOVA and calculated variance components for the heritability analysis of relative growth rate and triploidization success measured in diploid control and triploid pressure-shock family groups. *, $P \leq 0.05$; ** $P \leq 0.01$, ***, $P \leq 0.001$.

Trait	Source	Diploid Control				Triploid Pressure-Shock			
		SS	*df*	*MS*	*Var.*	*SS*	*df*	*MS*	*Var.*
Relative growth rate									
Rgr-c	Sire	0.334	28	0.012	5.0×10^{-5}	0.549	28	0.020	0.0006**
	Dam(sire)	0.309	29	0.011	0.001***	0.241	29	0.008	0.007***
	Offspring	0.769	522	0.001	0.001	0.755	518	0.001	0.001
Rgr-1	Sire	0.392	29	0.014	5.0×10^{-5}	0.435	30	0.014	0.001
	Dam(sire)	0.372	30	0.012	5.0×10^{-4}***	0.332	31	0.011	5.0×10^{-4}***
	Offspring	0.633	1142	0.001	0.001	0.703	1168	0.001	7.5×10^{-5}
Rgr-2	Sire	0.293	29	0.010	-2.5×10^{-5}	0.857	30	0.029	5.5×10^{-4}***
	Dam(sire)	0.331	30	0.011	5.0×10^{-4}***	0.231	31	0.007	3.0×10^{-4}***
	Offspring	1.028	1140	0.001	0.001	1.117	1176	0.001	0.001
Rgr-3	Sire	0.185	28	0.007	0.001	0.152	28	0.005	1.0×10^{-4}
	Dam(sire)	0.164	29	0.006	5.0×10^{-4}***	0.099	29	0.003	2.0×10^{-4}***
	Offspring	0.654	468	0.001	5.0×10^{-5}	0.558	483	0.001	0.001
Triploidization									
	Sire	N/A	N/A	N/A	N/A	26.091	28	0.932	0.0112
	Dam(sire)	N/A	N/A	N/A	N/A	15.016	29	0.518	0.025***
	Offspring	N/A	N/A	N/A	N/A	67.832	998	0.068	0.068

Table 2.5. Sire component heritability ($h^2 \pm SE$) and maternal effect estimates (expressed relative to total variance, V_m, %, and as an absolute value) for measured performance traits. Heritabilities considered significantly different from zero are marked as: *, $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Triploid $h^2 \pm SE$ values highlighted in bold type are significantly larger than comparable diploid values. NA = not applicable, UB = unbalanced, B = balanced, n = sample size, N/A = not applicable.

Trait	$h^2_{xire} \pm SE$		V_m relative, absolute		Design
	2N-Control	3N-Pressure	2N-Control	3N-Pressure	Dams, sires, n
Triploidization	NA	0.43 ± 0.20 (0, 1 scale)	N/A	13%	UB: 58, 29, 1056
	NA	0.32 ± 0.14 (underlying)			
Size at age					
W-1	0.50 ± 0.21	1.02 ± 0.35*	44%, 0.0140***	27%, 0.0122	B: 60, 30, 1200
W-2	-0.32 ± 0.10	-0.14 ± 0.29	51%, 0.452	60%, 0.686***	B: 60, 30, 1200
W-3	-0.01 ± 0.07	0.71 ± 0.29	51%, 0.634***	28%, 0.403	B: 58, 29, 580
W-4	0.18 ± 0.14*	0.80 ± 0.33*	38%, 0.894***	24%, 0.690	B: 58, 29, 580
Relative growth rate					
rgr-c	0.10 ± 0.11	1.04 ± 0.31**	46%, 0.00095	74%, 0.0064***	B: 58, 29, 580
rgr-1	0.13 ± 0.08	0.19 ± 0.11	29%, 0.00045	0%, -0.0005	B: 60, 30, 1200
rgr-2	-0.07± 0.02	1.19 ± 0.39***	33%, 0.0005	0%, -0.00025	B: 60, 30, 1200
rgr-3	0.13 ± 0.12	0.31 ± 0.18	0%, -0.0005	8%, 0.0001	B: 58, 29, 580
Serum lysozyme activity					
SLR-response to vaccination	-0.41 ± 0.10	0.08 ± 0.15	30%, 12.966***	31%, 7.974	UB: 36,18, 235

2.3.2.1 Size-at-age

While the component of variance in size-at-age associated with differences between the progeny of different sires (among sires component) was considerably smaller than that associated with differences between the progeny of different dams (nested within sires) at all sample points in both diploids and triploids, the among sires component of variance in triploids was on average four times larger than the comparable diploid value. A mean of 16% (15.78%) of the variance in size-at-age of triploid progeny and 4% (4.22%)of the variance in diploid progeny was attributable to differences among sires (Table 2.3). This result suggested that a larger proportion of the phenotypic variance in size-at-age of triploid progeny was due to additive genetic effects. As can be seen in Table 2.5, the estimates of additive genetic variance (as a proportion of total phenotypic variance) or heritability, were consistently higher in triploids at each sample point (except for W-2 when both estimates were negative); this translated to an average (over all size-at-age sample points) of 63% of total phenotypic variance explained by additive genetic variance in triploids versus 17% in diploids (calculated as the mean of 4 x among sire variance). Heritability estimates of size-at-age at W-1 and W-4 in triploids and W-4 in diploids were found to be significantly different from zero. However, the large standard errors associated with the heritability estimates for size-at-age result in 95% confidence intervals that rendered differences between diploid and triploid estimates predominantly non-significant. The proportion of the phenotypic variance in size-at-age attributable to differences among dams was surprisingly similar between ploidy types. Of total phenotypic variance at each of sample point (W-1, W-2, W-3 and W-4), 56%, 51%, 51% and 42% in diploids and 55%, 60%, 46% and 44% in triploids was attributable to differences among progeny of females mated to the same male suggesting that considerably less variance was associated with

maternal, dominance and epistatic effects in triploids than in diploids. Maternal effects estimates (relative, V_m, %) for size-at-age were substantially lower for triploids than for diploids at each sample point (except at W-2;Table 2.5) and were on average 11% lower than diploid estimates. The absolute value of the variance attributable to maternal effects was found to be significantly higher in diploid groups than in triploid groups as tested with Pitman's procedure for correlated populations, although this trend was reversed at W-2.

2.3.2.2 Relative growth rate

The distribution of variance in relative growth rate was somewhat more complex when considered as three distinct periods of growth (rgr-1, rgr-2, rgr-3) than when overall relative growth rate was considered (rgr-c). Similar to the trend in size-at-age, substantially more variance in the overall relative growth rate was attributable to differences among progeny of different sires (among sire variance) in triploids than in diploids. Among sire variance accounted for 7% of total phenotypic variance in triploids and only 2% in diploids; this resulted in a much larger additive genetic variance component in triploids than in diploids as is evident by the heritability estimates for rgr-c in Table 2.5 ($h^2 \pm$ SE, 2N = 0.10 $\pm$ 0.11; 3N = 1.04 $\pm$ 0.31). Only the triploid complete relative growth rate heritability estimate was found to be significantly different from zero. While large standard errors again made diploid and triploid heritability estimates not significantly different from each other, there is a distinct indication of greater genetic determination of relative growth rate in triploids than in diploids. The distribution of variance among dams (within sires) and among progeny (within dams) was substantially different between ploidy types in terms of magnitude (2N; 49% among dams, 49% among progeny; 3N: 81%% among dams, 12% among progeny) suggesting

that there was more intra-family variation in diploids but potentially more influence of dominance, epistasis and maternal effects in triploids than in diploids. The relative maternal effects estimate (V_m, %; Table 2.5) confirmed the substantially higher influence of the maternal effects component in triploids for this trait. The relative value of maternal effects for triploids was 28% higher than that of diploids (2N-V_m = 46%, 3N- V_m = 74%). Absolute variance attributable to maternal effects in triploids was significantly higher in triploids than in diploids ($P < 0.001$).

When relative growth rate was considered as three distinct periods of growth (rgr-1, rgr-2, rgr-3), diploids and triploids exhibited opposite temporal trends in the distribution and magnitude of variance components over the study period (Table 2.4). In diploids, the among sire component of variance accounted for 3% and 0% of the total phenotypic variance in growth periods rgr-1 and rgr-2 and rose to 65% of the total phenotypic variance during rgr-3, just prior to saltwater transfer. The opposite trend in among sire variance was exhibited by triploids. Triploid among sire variance accounted for 63%, 30% and 8% of the variance among progeny of different sires in rgr-1, rgr-2 and rgr-3. The among dam component of variance remained stable in diploids accounting for between 32-33% of the total phenotypic variance during each growth period while the triploid among dam component declined (accounting for 32%, 16% and 15% of the total phenotypic variance in each growth period). Opposite trends in the among progeny (within dam) component of variance were also apparent between ploidy types, with diploid variance decreasing and triploid variance increasing over time.

Relative maternal effects estimates (V_m, %; Table 2.5) were substantially lower in triploids than in diploids for each growth period when considered separately (averages of 3%-3N and 21%-2N). Maternal effects estimates for diploids initially increased and then declined over the experimental period (rgr-1 = 29%, rgr-2 = 33%, rgr-3 = 0%; Table

2.5) while triploid estimates were essentially zero (rgr-1 and rgr-2) and then experienced a slight increase (rgr-3) (rgr-1 = 0%, rgr-2 = 0%, rgr-3 = 8%; Table 2.5). Absolute maternal effects variances were either negative or very low (Table 2.5). Pitman's test for significant differences in variance could not be used due to negative or zero variance values. The general trends in variance distribution suggest that triploid relative growth rate was becoming more variable over time while diploid variability declined. Additive genetic variance appeared to be quite variable in triploid groups over the relative growth rate periods and remained fairly constant for diploid groups, but all heritability estimates for these growth periods, at least for diploids were not significantly different from zero. Heritability in triploids was significantly different from zero for rgr-2; this estimate was also significantly different from the diploid heritability estimate for this period ($h^2 \pm$ SE, 2N, 0.07 ± 0.02; 3N, 1.19 ± 0.39).

2.3.2.3 Response to Vaccination: Serum Lysozyme Activity

In contrast to the growth parameters detailed above, the phenotypic variance of serum lysozyme activity response to vaccination (SLR; Table 2.3) was distributed similarly in diploids and triploids. There was no variance associated with differences among progeny of different sires in diploids and only 2% of total phenotypic variance was associated with the among sire component in triploids. The remaining phenotypic variance was predominantly distributed at the inter-individual level so that 70% (diploid) and 65% (triploid) of the total phenotypic variance was associated with differences among individual progeny within families. Variance attributable to among dam differences accounted for the remaining variance, with 30% and 33% associated with the among dam component in diploids and triploids respectively. Heritability estimates of the difference between pre- and post-vaccination lysozyme activity (the response to

vaccination) in diploids and triploids were not significantly different from zero and were not significantly different from each other since 95% confidence intervals overlapped (h^2 ± SE, 2N, -0.41 ± 0.10; 3N, 0.08 ± 0.15). While the relative maternal effects estimates of the response to vaccination were not different (2N = 30%, 3N = 31%: Table 2.5), absolute variance attributable to maternal effects was significantly higher in diploids than in diploids (Table 2.5).

2.3.2.4 Triploidization Success

As with the serum lysozyme acitivity response to vaccination, variation in triploidization success was partitioned mainly among progeny (within dams) (65%) and among dams (within sires) (24%). Although more variance was associated with differences among progeny of different dams bred to the same sire than with differences among progeny of the same sire, 11% of the total phenotypic variance was associated with the among sire component suggesting that a substantial proportion of the variability in triploidization success was under additive genetic control. The underlying heritability estimate, while not associated with a significant sire effect (in the ANOVA) was moderate (h^2 ± SE, 0.32 ± 0.14) and the 95% confidence interval did not encompass zero. Maternal effects were present but were relatively low (13%-relative estimate; absolute variance = 0.0138) suggesting that the combined environmental and genetic components of the effect of the maternal phenotype, dominance and epistatic effects on triploidization of offspring was not large.

2.3.2.5 Sign Test of Heritability Differences

Triploid heritabilities were consistently higher than those of diploids and the sign test confirmed this by rejecting the null hypothesis that the median difference between

triploid and diploid heritability estimates was zero [$P(X \leq 0 \text{ or } X \geq 9) = 0.0019$; $C_{0.05(2),9} = 1$, $n\text{-}C_{0.05(2),9} = 8$; $0.02 > P > 0.001$].

2.3.3 Performance and Triploidization Success

As performance-based differences between treatment groups were not the main focus of this paper, I review these results only briefly below. As was found in Chapter One, diploid families outperformed triploids but the differences were minimal. While triploids experienced survival that was 13-12% lower than that of diploids during incubation and after ponding, relative growth rate over the freshwater rearing period did not differ significantly between treatment groups. By the time of saltwater transfer, mean weight of diploids and triploids was also not significantly different. Additionally, no significant difference in the non-specific immune parameter, serum lysozyme activity was found either within or between ploidy types, before or after immersion vaccination. Pre-vaccination lysozyme activity however was lower than post-vaccination activity for both diploid and triploid family groups although levels after vaccination were not significantly higher than before exposure to the vaccine. Interestingly, while the overall mean lysozyme activity level of pressure-shock triploid family groups was 3% higher than that of diploid control groups prior to vaccination, it was 10% lower than that of the control groups after vaccination, but not significantly so.

Overall triploidization success was 88%. A small percentage of samples (n = 21 fish or 1.0% of those analyzed) were discarded because of uncertainty in the determination of ploidy due to overlap between the diploid and triploid mean red blood cell nuclear length distributions. Family-specific analysis of induction success indicated that triploid levels ranged from 22% -100%, with 71% of the 62 families (44/62) having triploidization rates between 95% (13 families) and 100% (31 families). Of the remaining

families, 7 had triploidization rates between 80-89%, 5 between 71-78%, 2 between 65-68%, 3 between 30-40% and 1 family had a triploidization rate of 22%. The presence of diploids in samples (up to 78% in one family) is an acknowledged limitation of this study. However, all known diploids were dropped from analysis of size-at-age at the W-3 and W-4 sample points (~235 and 244 days post-fertilization) as well as the respective relative growth rate sample periods and only known triploid and diploid samples were included in the serum lysozyme activity level assays, so that the inclusion of pressure-shocked but diploid individuals in analysis was only confounded with survival and the W-1 and W-2 (~159 and 215 day post-fertilization) size at age measurements.

2.4. Discussion

Triploidization is expected to increase total phenotypic variance. Using a quantitative genetic framework, the total phenotypic variance can also be decomposed into genetic effects (i.e., additive, dominance and epistatic components) and environmental effects (e.g., a maternal effects component). However, the specific experimental design used in this study, a paternal half-sib design, can only decompose total variance into an additive genetic component versus a combined epistatic, dominance and maternal effects component. Despite this limitation, the differences between the patterns of distribution of the phenotypic variation are useful and can provide insight into the relative importance and distribution of the casual components.

The observed increased phenotypic variability among, and to a lesser extent, within, triploid families shows that the addition of an extra set of chromosomes directly affects the resulting phenotypic variance. Among family variance was expected to be larger than within family variation because of the 2/3rds maternal genetic contribution. This phenotypic effect is probably most evident because environmental variance has

been minimized and genetic composition of families has been kept constant. The expansion of phenotypic variance might be explained within a developmental context, especially if the developmental process is thought of as a set of modules consisting of networks of interacting transcriptional genes. If these networks have evolved to be increasingly complex as a result of selection for developmental stability (Frank 1999; Siegal and Bergman 2002), with the suppression of genetic variation (cannalization) as a by-product (Wagner *et al.* 1997), then perturbing the regulatory control of development by forcing triploidization could release phenotypic variation (Stearns 2002). While increased phenotypic variation was evident in growth related traits in triploids, significantly less variation occurred in the triploid lysozyme response to vaccination than was evident in diploids. It is unknown why cannalization appears to have been disrupted in the growth-related parameters but not in the immune parameter. It is possible that this relates to differences in the regulatory control complexity and/or parental specific genetic expression patterns of growth-related and non-specific immune genes. Significantly higher among and within family phenotypic variance in growth parameters (of length and weight) was also recently noted for triploid full-sib Atlantic salmon families (Friars *et al.* 2001). The authors attributed the higher levels of variance to a triploidization-induced disruption of uniformity but did not hypothesize a mechanism. Phenotypic variation of immune parameters has not been looked at in detail before.

Total phenotypic variance was also partitioned into among sire, among dam and among progeny components for the heritability analysis. Among sire variance is used to estimate additive genetic variance and heritability while the difference between the among dam and among sire variance is used to estimate the combined contribution of dominance, epistasis and maternal effects. Additive genetic variance and heritability are

important because they describe the genetic resemblance between relatives and can be used to estimate the short term response to selection (Lynch and Walsh 1998; Wang *et al* 2002); however such considerations are irrelevant in triploids because of sterility. However, because additive genetic variance also reflects the variance of the average effect of alleles of the parents as expressed in the offspring (Falconer and Mackay 1996), these estimates can provide information about the genetic architecture and gene expression patterns of triploids.

Heritability estimates of performance parameters in this study were significantly higher in triploids than in diploids (Table 2.5; sign test results); this is clearly illustrated in Figure 2.1 as the difference between triploid individual trait heritability values and the mean diploid heritability value (over all traits). A simple increase in genetic material should cause significant changes in the amount of genetically controlled phenotypic variance if there is an overall additive relationship between the transcription of alleles and their affect on the phenotype. If dosage compensation does not occur, higher heritabilities in triploids for growth traits that typically show moderate heritability values in diploids (e.g., Gjedrem 1983; Kinghorn 1983) would be consistent with the occurrence of an overall ploidy dependent regulation (i.e., positive gene dosage effects) of gene expression averaged over all growth related loci.

While an increase in genetic content may cause higher heritability values in triploids, it must be kept in mind that an inflation of heritability might also occur because of an increase in epistatic interactions (i.e., additive x additive or the interaction between homozygous loci). This is possible because the covariance between half-sibs, which is the most statistically valid component with which to estimate heritabilty (as it contains 1/4 of the phenotypic variance attributable to the inherited action of genes; i.e. the additive genetic variance) also contains 1/16th of the variance attributable to additive by

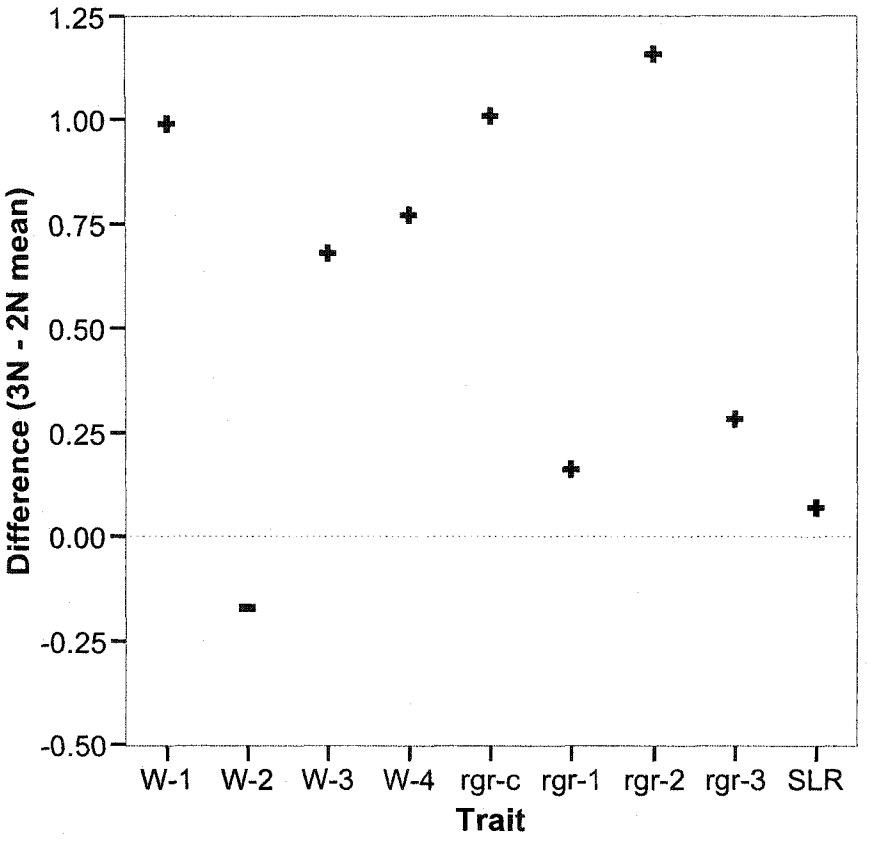


Figure 2.1. Difference between mean diploid heritability (for all traits combined) and individual performance trait heritability estimates of triploids. Differences are marked by a plus sign if the value of $h^2_{triploid}$ > the $\overline{h^2}_{diploid}$ value and by a minus sign if $h^2_{triploid}$ < $\overline{h^2}_{diploid}$. W-1 to W-4 represent weights, rgr-c, and rgr-1-3 represent relative growth rates and SLR represents serum lysozyme activity response to vaccination. See Table 2.1 for details.

additive epistasis (Falconer and Mackay 1996; Roff 1997). This interaction is generally ignored because it is assumed to be very small (Falconer and Mackay 1996; Roff 1997). However, if in triploids there is a higher level of additive-by-additive interaction between loci this might result in inflated heritability estimates especially since the additive effect

of a gene will change dependent on the frequencies of its epistatic partners (Cheverud *et al.* 1999; Wade 2002).

Maternal effects are defined as the non-genetic influences of the maternal phenotype, genotype, and environment on the phenotype of the offspring (Mousseau and Fox 1998; Falconer and Mackay 1996). Maternal effects in chinook salmon are mainly transmitted through prezygotic allocation, since there is negligible maternal care. For example, the mother's nutritional status, hormonal ovarian environment or susceptibility to disease may affect egg quality (e.g.,yolk nutritional value, mRNA or maternal protein content, organelle metabolism), egg size, meiotic status at fertilization and juvenile mortality (Rossiter 1996; Heath and Blouw 1998; Wade 1998).

Maternal effects are estimated in a paternal half-sib experimental design by subtracting the among sire variance from the among dam (nested within sire) variance and expressing it as a proportion of the total variance (Falconer and Mackay 1996). However, because the among dam component of variance includes dominance and specific proportions of the variance due to epistatic interactions (i.e., interactions between alleles, as well as interactions between homozygous loci, heterozygous loci and interactions between homozygous and heterozygous loci) and all of the variance due to maternal effects (in addition to 1/4 of the additive genetic variance) (Falconer and Mackay 1996), this estimate will be inflated. Although the among dam variance is therefore not considered useful for estimating the narrow sense heritability, it may reflect the relative importance of specific epistatic interactions and can be used to detect the presence of maternal effects, albeit confounded with dominance and epistatic effects. However, because the epistatic effects included in the among dam component of variance are generally considered to be quite small in diploids, they are usually

ignored (Falconer and Mackay 1996; Roff 1997). Such an approach may not be entirely appropriate for triploid offspring, since the relative contribution from dominance and epistatic effects is simply unknown; however, maternal effects are known to be very large for diploid chinook salmon fry and are expected to swamp the non-additive genetic variance components during early development (Heath and Blouw 1998; Heath *et al.* 1999).

Both relative and absolute maternal effects estimates were found to be generally lower for triploids than for diploids for most performance traits (Table 2.5; note the w-2 and rgr-c exceptions). In Figure 2.2, the mean maternal effects value for diploid traits (all traits combined) is subtracted from individual triploid maternal effects values. This is especially interesting for a number of reasons. First, while the addition of 2 maternal sets of chromosomes would tend to increase the maternal additive component, it might also inflate or magnify the influence of maternal effects and thus potentially have a greater influence on offspring phenotype. This does not seem to have occurred. Second, because triploids have higher among sire values it indicates that dominance, epistasis and maternal effects have less effect on the triploid phenotype. However, if total phenotypic variance is increased in triploids but the combined contribution of maternal effects, dominance and epistasis remains relatively stable then the maternal effects estimate would be expected to be lower. Absolute maternal effects variance values (independent of total variance) in triploids however, exhibited a trend towards lower values (Table 2.5).

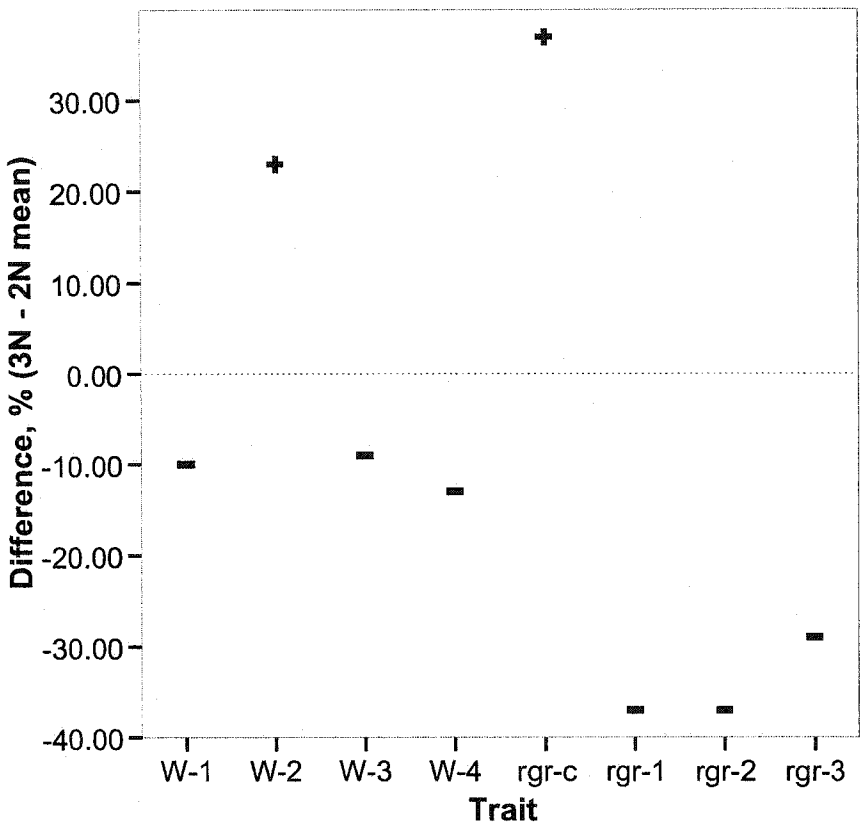


Figure 2.2. Difference between individual performance trait maternal effects estimates of triploids and the mean value of diploid maternal effects (for all traits combined). Differences are marked by a plus sign if the value of $h^2_{triploid}$ > the $\overline{h^2}_{diploid}$ value and by a minus sign if $h^2_{triploid}$ < $\overline{h^2}_{diploid}$. W-1 to W-4 represent weights, rgr-c, and rgr-1-3 represent relative growth rates and SLR represents serum lysozyme activity response to vaccination. See Table 2.1 for details.

Growth traits and survival of young diploid salmon are strongly influenced by maternal effects during the egg, larval and early juvenile stages (Kinghorn 1983; Heath and Blouw 1998; Heath, Fox, and Heath 1999; Nagler *et al.* 2000). However, the magnitude of maternal effects on offspring growth in farm-reared chinook salmon decrease as juveniles develop, becoming negative in a compensatory manner, and then

become not significantly different from zero by approximately 150 days post-fertilization (Heath and Blouw 1998; Heath, Fox, and Heath 1999). If the difference between sire and dam variances is mainly a result of dominance and epistatic genetic effects rather than predominantly maternal effects, the results suggest that these influences are less in triploid offspring. This is unexpected since increased structural complexity in triploid genomes is expected to increase the phenotypic effects of dominance and epistasis. However, if the main effects of triploidy on phenotype are additive and magnified due to the increased copy number of nuclear genetic material, the relative effects of dominance and epistatic interactions might not be detected if they are not as drastically influenced by triploidization.

Another interesting result of this study was that triploidization success was shown to have a moderate genetic basis ($h^2 \pm SE = 0.32 \pm 0.14$). This is the first estimate of heritability reported for this trait and it suggests that there is a genetic component to the cellular response to pressure-shock. This means that a breeding program to improve triploidization success might be successful; however the logistics would be difficult as a proportion of each spawned family would have to be retained as diploids to serve as broodstock.

The distribution of variance among causal components in the sib-analysis of triploidization success was unexpected as the majority of variance was attributable to differences among progeny (65%) rather than differences among dams (24%). This was surprising as the eggs from both females bred to the same sire were subjected to the pressure-based triploidization treatment simultaneously. If inter-individual variation within dams was predominantly responsible for whether or not triploidization by pressure was successful then it may mean that differences in the susceptibility of individual eggs to polar body retention within females exist. Whether this is due to

differences in intra-female timing of meiosis is unknown. Family differences in triploidization success of salmonids has often been noted and is most likely due to the combination of genetic differences between dams and environmental effects. For example genetic differences in meiotic timing, microtubule structure or egg provisioning might exist or interact with environmental factors such as differences in pre-spawning rearing environments of females, the timing between ovulation and spawning or the consistency of triploidization treatment (Levanduski *et al.* 1990; Diaz *et al.* 1993; Teskeredzic *et al.* 1993; Galbreath and Samples 2000).

In summary, triploidization increased phenotypic variation of growth traits both among and within families of chinook salmon. Additionally, the data clearly indicated that the proportion of phenotypic variance attributable to the average additive effects of alleles (additive genetic variance and heritability estimates) increased after triploidization while the relative size of dominance, epistatic and maternal effects probably did not. This pattern of variance distribution indicates that the primary effects of adding an extra set of chromosomes to the salmonid genome are additive and this, in turn is highly suggestive of a predominantly ploidy-dependent mode of gene expression. Dosage effects appear to be present in triploids; and specific gene expression patterns should show a general up-regulation in triploids.

In terms of the utility of triploidy for aquacultural purposes, triploidization appears to disrupt the normal inheritance of performance gains made through the selective breeding of diploids. This decreases the utility of triploids because the trend of increased phenotypic variation makes the prediction of performance difficult. It is recommended that individual fish farms weigh the potential ecological and marketing benefits of producing sterile stock with the disadvantages of producing a genetically less reliable animal for market.

General Conclusions

Triploidization is an abrupt and traumatic genetic perturbation that causes profound changes in genome and cell size. These changes appear to directly affect the phenotype of chinook salmon by increasing the range of performance responses. While this expansion of phenotypic variance does not result in better performance than diploids, the results of this study suggest that performance is not substantially compromised either, at least in terms of growth and immune response. Increased phenotypic variance however, translates into an increase in additive genetic variance indicative of an overall ploidy dependent pattern of gene expression. Triploidization was also accompanied by an apparent decrease in the relative influence of dominance, epistasis and maternal effects, which was unexpected because it was assumed that triploidization would result in a more complex genomic architecture.

Despite profound differences between diploids and triploids in the partitioning of phenotypic variance and potentially genetic expression, chinook salmon responded quite well to triploidization especially when pressure rather than heat was used for polar body retention. Growth as well as the specific and non-specific immune responses to vaccination was not substantially different between diploids and triploids. As expected, survival after triploidization was compromised but this occurred mainly during the embryonic and larval stages of development.

The obvious dichotomy between high and low performing families regardless of treatment/ploidy status and the existence of significant family components for many of the performance variables (Chapter One) indicate that selective breeding of diploids for increased triploid performance might be possible. However, the presence of family by treatment interactions (although explaining a relatively low amount of variance) observed in the Chapter One study and the increased range of phenotypic variance and

profoundly different pattern of variance partitioning found in the Chapter Two study suggest that the effectiveness of a selective breeding of diploid stock to increase triploid performance might be limited by prediction difficulties.

These studies have contributed to salmonid research by providing:

1) the first comparative quantitative genetic analysis of a vertebrate triploid model
2) the first heritability estimates for a triploid vertebrate
3) the first estimate of the heritability of triploidization success and,
4) the first comprehensive comparative assessment of triploid chinook salmon performance

The results described in this thesis suggest a number of areas for future study. Primarily, this would involve detailed marker based analysis followed by a survey of gene expression patterns using microarray technology. An ongoing study of this nature is currently underway involving a team of researchers from the University of Windsor, UNBC, UBC and DFO, funded by NSERC-CRD. A detailed comparison of diploid and triploid non-specific and specific immune parameters and expression patterns would be especially interesting. Additional research utilizing a more extensive breeding design for quantitative genetic analysis (e.g., factorial, partial diallel or Eisen design), while logistically difficult, would also be useful for separating and estimating the genetic and environmental influence of maternal and epistatic components.

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