5. DISCUSSION

Genetically improved forms including GMOs, are developed for a specific set of environmental circumstances in which they provide an advantage. However, in nature such genetically distinct forms may legitimately be regarded as mutant forms of the wild type. A considerable knowledge about genetics indicates that the probability of survival of mutant forms is extremely low because they are disadvantaged in viability and/or fertility under natural conditions. Thus, for example, in the genetically distinct farmed Atlantic salmon in Norway the males are very much less successful than wild males in securing mates. The survival rate of manipulated animals is also dependent on the technique applied (Beardmore, 2003). For transgenic fish with a growth hormone (GH) gene construct there are many studies conducted. For example GH-transgenic salmonids can display altered skeletal structure, reduced disease resistance, altered gonadal development, altered gut, gill, muscle heart and liver (Devlin et al. 2006).

Undoubtedly the main advantage of producing triploid fish is that this technique guarantees 100% sterile animals, and is the most effective (and economically viable) method for a large-scale animal production. Sterility protects somatic growth, survival and flesh quality from the negative effects of sexual maturation. In the long term, the genetic and ecological impact of interactions between wild and cultured fishes is limited by induction of triploidy and this minimizes environmental and human health concerns (Maxime 2008; Devlin and Nagahama 2002; Rasmussen and Norrisey, 2007). The information available on the survival rate of polyploids is contradictory and according to Teuer (2003) triploids have reduced survival rates and high rates of skeletal deformity, although Rasmussen (2007) states that polyploidy can result in enhanced growth and survival rates.

In the present study diploid and triploid rainbow trout were compared to assess the impact of triploidy on viability and development and their response to stressors. At the beginning of biological trials with diploid and triploid trout a

decrease in survival rate of triploids was observed (30 % TC and ~ 20% TA of mortality rate, figure 4.1) which was most notable in the first 14 days of the experiment (before hatching) and revealed an intrinsic fragility in the initial developmental stages associated with chromosome set manipulation. It is unclear if the reduced viability of triploid trout is related to changes in the vitellogenin content of eggs. With the diploid trout the survival at the beginning of the experimental trials was high, although viability decreased at 84 days after start feeding in the control group and at 140 days after the start of feeding in the diploid group fed diet with reduced phosphorus content (figure 4.1). According to the results of skeletal ontogeny it appears that the entire body axis is affected, these differences are visible from the exterior and internal structures were also modified. These results raise questions about the impact of an extra chromosome set and modified mineral availability on the normal patterning during development of the skeleton and also the molecular factors which may enhance the change or be associated with the change. A recent report in which a comparative study was carried out on the survival and growth of diploid and triploid fishes suggests that when diploid and triploid trout are reared in separate tanks, none of these indicators of fish health are ploidy-affected but rather result from tank effects (Maxime, 2008).

Dramatically enhanced growth rates in GMO's, with overexpression of GH can result in pleiotropic effects in addition to enhancing growth rates. In some cases, expression can be at a level which results in reduced viability and morphological abnormalities resembling acromegaly. Effects on disease resistance, metabolism, endocrine system, swimming ability, organ structure, and behaviour have also been observed in several species (Devlin et al., 2004). The growth performance of triploids which has been reported in the literature (Tiwary et al, 2005) indicates a high level of species specific variability. On the other hand according to the analysis of Maxime (2008) it appears that growth rates of triploid fishes are inconclusive and contradictory results about growth are frequently reported, even between individuals of the same species reared under similar conditions. Other finding indicate that triploidy in species such as rainbow trout, sunshine bass (a hybrid), coho salmon, and Atlantic salmon tend to grow poorly compared to their diploid counterparts (Tiwary et al 2004). In

the present study growth trials lasted 168 day or 219 day post fertilization, and led to the conclusion that at the mid-point of experiments triploid trout grew faster but then diploid trout by the end of the experimental trial this trend this was inverted and overall the diploid trout grew significantly more. The preceding results were based on measurement of standard length (figure 4.10) and also allometric associations - like the ratio between head length and vertebral column length (figure 4.11) which were always higher in diploids than triploids at the end point of the experiment. Other significant differences are biometric differences associated with meristic counts, like the total number of vertebra (figure 4.8) with generally triploids having one less vertebra than diploids; or the total area of vertebra that differ according to body region (figure 4.9) and that despite initial differences in early stages (107 - 163 dpf) in which triploid trout had a greater vertebral area than diploid trout in some body regions, in later stages diploids had the highest vertebral area in all regions considered. All results indicated high variability in biometric parameters for both diploid and triploid trout irrespective of dietary modification and despite this variability by the end of the trial in the juvenile stage triploids were smaller than diploids in all parameters.

Even with divergences in growth in reviews by Maxime (2008) and Rasmussen (2007) states that triploidization is recognized as the most practical, economical and effective method for a large-scale production of sterile fishes. The prime advantage of triploids which outweighs the disadvantages is the reduction in the genetic and ecological impact of interactions between wild and cultured fishes. There is also less legislation regarding the creation of triploid animals compared to transgenic animals when legislation controlling their production is considered across Europe (EU). In general it is considered that any fish produced by induction of triploidy are not genetically modified organisms (Directive 2001/18/EC European Parliament – Article 2 – Annex IA – part 2). All these factors, even when the reduced growth rate of triploids compared to diploids is considered favours production of triploids as they do not loose quality associated with reproduction and also do not represent a risk of genetic contamination should they escape into the environment.

Comparative data about the body composition of triploid and diploid fishes available in the literature are very limited. Most of the work done relies on the muscle content, visceral fat and lipid content. According to Roy et al. (2002) different mineral diets, namely P deficient diets cause a delay in bone mineralization. Vielma and Lall (1998) state that there is an apparently higher efficiency in absorption in P deficient fish than in P replete fish, suggesting that fish might have similar mechanisms to mammals which in hypophosphatemia stimulate mechanisms which lead to increased intestinal absorption of P. In the present study a detailed analysis of the developing skeleton of diploid and triploid trout was carried out in response to changes in phosphorus availability in the diet. From the data obtained it appears that the levels of P and Ca in young larvae are very different at the initial stages of development and dietary restrictions have a variable effect (figure 4.2). Whole body Ca levels have a tendency to increase as larvae grow and develop, even in larvae maintained on a P poor diet (A). The whole body concentration of P is higher than Ca from early stages even when it is of reduced availability in the diet. Evaluating the P content and comparing the first samples analyzed (51 dpf) in all experimental groups with subsequent stages only a small increase occurs. In subsequent developmental stages there is a tendency to stay within constant limits (9 µmol P/mg Ash) and no statistical differences in P content were found between samples. Calcium content is very significantly different in the beginning of the feeding trials (2 months after exogenous feeding) between triploids fed a normal or modified diet and diploid fed a control or modified diet. At 107 dpf no significant differences appeared in the Ca content between the different experimental groups, possible due to the fact that at this age the skeleton is completely formed. All these quantitative observations of variation in mineral content associated with dietary status are corroborated by the diagram at figure 4.5, where it is clear that diploid (C and A) and triploids (C and A) present similar overall pattern of ossification. However, variations are evident and in the TC group the cleitrum, rays, epurals and the urostyle are the first structures to ossify while in the DC group the vertebra and the pleural ribs started ossifying earlier. In this study in agreement with other studies it is observed that the influence in phosphorus availability is determinant, and P reduction causes a delay in the timing of the completion of skeletal ontogeny. It appears

that dermal structures are the most affected by the mineral deficient diet and their appearance is significantly delayed (figure 4.6 and 4.7, table 4.3). Together the results for mineral content and skeleton ontogeny reveal that overall the skeleton formation during trout development is more affected by P depletion than Ca depletion. At the last sample point 219 dpf, and after animals had the opportunity to recover from mineral depletion by offering diets with a normal mineral content, data revealed that the Ca and P content of vertebrae from different skeletal regions was very variable. Diploid animals fed with diets deficient in minerals and subsequently fed with diets containing an adequate mineral content initially overcompensate and are able to incorporate higher levels of calcium into bone. The skeleton of fish therefore appears to possess an optimal level of minerals and when a shortfall occurs this is "sensed" and increased mineral incorporated when it is possible allowing the skeleton to recover.

Other internal differences were also registered in the literature comparative studies of triploid and diploid Salmonidae, revealed a lower density of muscle satellite cells in triploid fish which was associated with reduced hyperplasia (Suresh and Sheehan 1998; Johnston et al. 1999). Trangenic coho salmon containing growth hormone gene construct was observed an increased growth rate, resulted in changes in muscle architecture consistent with increased rates of hyperplasia (Hill et al. 2000). The reduced number of myocytes in triploids is compensated by their rates of hypertrophic growth which is greater than diploids (Johnston et al. 1999). The results of the present study are in general agreement with previous studies and the myotome number was significantly lower in triploids on a phosphorus poor diet compared to triploids fed a normal diet and the later groups had less myotomes than either of the diploid groups at 64 dpf, although variation occurred associated with developmental stage (cause at these age, animals have no diet restrictions). The present study indicates that ploidy manipulations affected myotome number and that this was even more significantly affected when it was combined with a modified diet.

The skeletal markers analyzed in this study where Osteocalcin (Osc), Osteonectin (Osn) and Osteopontin (Osp). Which are proteins of the

extracellular matrix important for skeletogenesis and calcification. Osn is a secreted calcium binding protein involved in bone development that has affinity for hydroxyapatite and collagen (Gadeau et al., 2001). Osn is present in the extracellular matrix of tissues that have a high turnover rate, such as bone (Estevão et al., 2005). In skeleton biology, osteonectin has been described as a positive factor in the mineralization process as well as in osteoblastic cell lineage differentiation and is downregulated by the hypercalcemic hormone PTH (Estevão et al. 2005; Redruello et al., 2005). Osp is an acidic phosphoprotein which contains a hydroxyapatite binding site and it is suggested that this protein plays a role in calcium metabolism and potentially in the calcification process. Osc is a vitamin K-dependent matrix protein and in vitro it strongly inhibits calcium salt precipitation and shows a strong affinity for hydroxyapatite, but inhibits crystal growth by delaying nucleation. Although its role is still unclear, it has been demonstrated that Osc normally limits bone formation (Ducy et al. 1996; Gadeau et al., 2001). It should be noted, that transcripts of Osc, Osp and Osn and not protein were measured in the present study and also that this measurement occurred 4 weeks after the start of experimental manipulations. It is perhaps unsurprising therefore that no significant differences were observed in transcript number between any of the experimental groups in the study, presumably because equilibrium had been established. Alternatively the modification in available Ca and P during bone formation was insufficient to cause a modification in gene transcription. Moreover, as the genes were quantified in extracts containing a number of different tissue, eg. muscle, nerve, skin, blood vessels in addition to skeletal tissue, this may have rendered the method less sensitive to small tissue specific changes. An interesting observation was the remarkably small variation observed in gene transcription between individuals in each experimental group, it is tempting to speculate that this may be indicative of genes with a generally high constitutive expression. It will clearly be of importance in the future to analyze Osn, Osc and Osp in other experimental groups when significant modifications in skeletal development or calcium homeostasis are occurring.