THE ZEBRAFISH *DANIO RERIO*; A PISCINE MODEL FOR BIOTECHNOLOGY

by

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For Daphne, Lorraine and Ivan

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ABBREVIATIONS

α	alpha
β-Gal	β-Galactosidase
χ	gamma
°C	degrees Centigrade
%	percent(age)
μg	microgram(s)
μm	micrometre(s)
μl	mocrolitre(s)
~	approximately
2N	diploid chromosome number
3N	triploid chromosome number
4N	tetraploid chromosome number
AI	artificial insemination
ave	average
BKD	bacterial kidney disease
bp	base pair(s)
BSA	bovine serum albumin
cat	chloramphenicol acetyl transferase gene
CAT	chloramphenicol acetyl transferase enzyme
cm	centimetre(s)
cpm	counts per minute
DNA	deoxyribonucleic acid
eg	for example
ES cell	embryonic stem cell
et al	et alia (and others)
g	gram(s)
GD	golden danio

GH	growth hormone(s)
GSI	gonado-somatic index
GV	germinal vesicle (stage)
GVBD	germinal vesicle breakdown (stage)
h	hour(s)
HBSS	Hank's balanced salt solution
HR	homologous recombination
HS	heat shock
ICM	inner cell mass
ICM	inner cell mass
IHN	infectious hemopoietic necrosis
IHNV	infectious hemopoietic necrosis virus
kPa	kilo pascals
L	litre(s)
lacZ	β-Galactosidase gene
LD	leopard danio
LN_2	liquid nitrogen
luc	luciferase gene
LUC	luciferase enzyme
mg	milligram
Milli-Q	Milli-Q reagent grade water
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
nm	nannometre(s)
OD	outer diameter
p(plural pp)	page(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
per comm	personal communication

pf	post fertilisation
pH	percentage of Hydrogen ions
pl	picolitre(s)
RO	reverse osmosis
RT	room temperature
sp/ml	sperm/ml
spp	species
TE	Tris EDTA
UV	ultra violet light
v/s	versus
x *g*	unit of gravitational field
ZD	zebra danio

ABSTRACT

This thesis reports on studies aimed at investigating the potential of the zebrafish *Danio rerio* to provide a convenient, fecund and cost efficient piscine model for biotechnology. The technical and biological limitations of the model are identified and ways of overcoming or circumventing these limitations explored in three areas of biotechnology.

The first area examined was transgenesis with the general aim of improving procedures for ***single gene manipulation***. Initial studies focused on reporter sequences for transgenes and detection methods suited to studies in fish. The fecundity of the zebrafish makes it possible to generate large numbers of experimental individuals and the need for an expression assay rather than DNA analysis to provide an early indicator of successful gene transfer was identified. Preliminary investigations revealed that the use of gene constructs containing genes of bacterial origin such as *lacZ* and chloramphenicol acetyl transferase (*cat*) was limited due to the background expression caused by bacteria present in the gut and aquatic environment. However, a transgene containing the gene coding for firefly luciferase (pGL2) proved to be satisfactory and formed the basis of the development of an extremely sensitive, rapid and cheap expression assay, with minimal background. Using this construct various gene transfer techniques were explored.

Transgenesis in zebrafish was first achieved following injection of 1-cell (zygote stage) embryos with pGL2, resulting in stage dependent variation in luciferase activity as assessed in individual embryos. Expression was first detected 5h post fertilisation (pf), followed by a sharp increase at 15h (somitogenesis) to peak at 24h, expression levels then declined returning to baseline values in Day 7 (168h) larvae.

The luciferase transgene was also used to demonstrate the feasibility of sperm mediated transfer in zebrafish. A gene transfer rate of 13-14% was achieved by this means as measured by analysis of both embryos and adults in the founder (F0) generation with a germline transmittance rate to F1 of 10-19%. Preliminary studies on sperm mediated transgenesis were conducted in the medaka (*Oryzias latipes*) using *lacZ* and *pGH* (porcine) constructs. A high gene transfer rate of 36% was found by analysis in Day 7 larvae, which was similar to the transfer rate achieved following microinjection into the germinal vesicle (GV) of maturing oocytes in the same species (33%).

The most successful procedures were those based on gene transfer to ovulated (metaphase II) oocytes. This novel route was made possible by the discovery that there were factors in seminal fluid that inhibited the spontaneous parthenogenetic activation which normally followed ovulation of zebrafish oocytes thus extending the time in which oocytes could be manipulated. Using this approach, a very high rate of gene transfer was achieved with the resulting embryos displaying significantly ($P<0.0001$) higher levels of expression of the pGL2 transgene at 24h pf, than embryos microinjected at the 1-cell (zygote) stage. The gene transfer rate for the oocyte injected group was also double that obtained for the zygote injected group when assessed at the adult stage (Adult F0), (47 and 25% respectively) with a greater proportion of the F0 transgenics, resulting from oocyte injection, transmitting the transgene to their progeny.

The second area explored was ***partial genome manipulation***. This study was initiated to provide a basis for an anticipated future need to create chimeric embryos as one means of reinstating embryonic stem cells within the germline. The study investigated the possibility of producing chimeras between two phenotypically distinguishable but related species of the genus *Danio* (zebrafish, *D. rerio* and leopard danio, *D. frankei*). It has shown that cells from blastula stage embryos can be successfully transplanted between embryos of these species to yield a high frequency of inter-specific phenotypic chimeras as

assessed both at the embryonic (88%) and adult (40%) stages. The chimeras were recognisable on the basis of the differences in pigment patterns between the species and thus could be used to provide a useful marker for identifying chimeras at both larval and adult stages. It was found that differences in the relative contribution that the donor blastomeres made to the resulting chimeras could be related to differences in the cleavage rate of donor and recipient embryos.

The third area investigated dealt with ***entire genome manipulation***. This study area was implemented as a possible means of addressing concerns about the potential biohazard that transgenic fish might pose, should they escape. Triploid fish have been shown in several studies to be sterile and temperature induced tetraploidy was explored as one means of controlling reproduction in the zebrafish by facilitating the production of triploid (3N) zebrafish (*via* a 4N x 2N mating). The first series of experiments using temperature shock to induce chromosomal doubling, resulted in a highly variable tetraploid induction rate (0-38%) necessitating the development of a non-invasive approach, based on cleavage patterns, which allowed tetraploid embryos to be identified early in their development. The method developed was a significant advance as it allowed identification of tetraploid embryos at the 4- to 8-cell stage, with 100% accuracy and facilitated optimisation of the parameters (temperature, duration, and initiation time) needed to induce tetraploidy. As a result of studies on over 6000 embryos, procedures were developed which yielded a consistent tetraploid induction rate of 49%; these were a temperature shock of 41 ±0.5°C, initiated at 12 min post fertilisation, administered for a 2 min duration. However only a small percentage (9% or 64/736) of the tetraploid embryos developed to the larval stage where they underwent swim bladder inflation and began feeding. Furthermore, all these larvae were sub-vital when compared with controls, with the reduced vitality becoming more pronounced with age; no tetraploid zebrafish survived beyond 50 days of age.

In summary the data provided in this thesis clearly establish the zebrafish as a potentially valuable model for a range of biotechnical applications such as transgenesis, production of chimeras and chromosome set manipulation. Several unique contributions are identified including, (1) demonstration that gene transfer is best done using oocytes rather than zygotes as it results in increased rates of gene transfer and decreased incidence of mosaicism in F0 transgenic zebrafish, (2) that sperm mediated transgenesis is feasible and offers a practical way of treating large numbers of gametes, (3) that it is possible to create recognisable chimeras between two closely related but phenotypically distinct species, and (4) show that the relative contribution of donor blastomeres to chimeras can be varied by exploiting the differences in cleavage rate between donor and recipient embryos, and finally (5) developing non-invasive means of identifying tetraploid individuals early in development. These developments pave the way for a range of future investigations in basic cellular and molecular embryology in the zebrafish and establish this species as a useful model for biotechnological applications to aquaculture.

DECLARATION

The experimental work described in this thesis was conducted in the Department of Obstetrics and Gynaecology, University of Adelaide, South Australia, during 1991-1995. I hereby declare that this thesis contains no material which has been submitted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, no material described herein has been previously published or written by another person, except where due reference is made in the text.

If accepted for the award of a Ph.D. degree I consent to this thesis being available for loan and photocopying.

Paul J Verma

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Bawden, C.S., Sivaprasad, A.V., Verma, P.J., Walker, S.K. and Rogers, G.E. (1995). Expression of bacterial cysteine biosynthesis genes in transgenic mice and sheep: towards a new in vivo amino acid biosynthesis pathway and improved wool growth. TRANSGENIC RESEARCH, 4, 87-104

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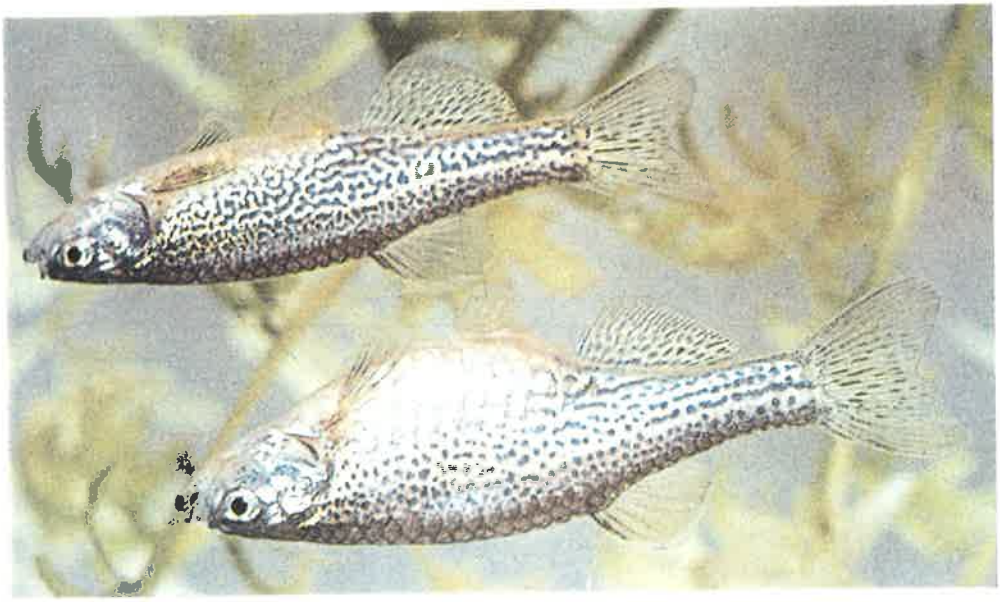
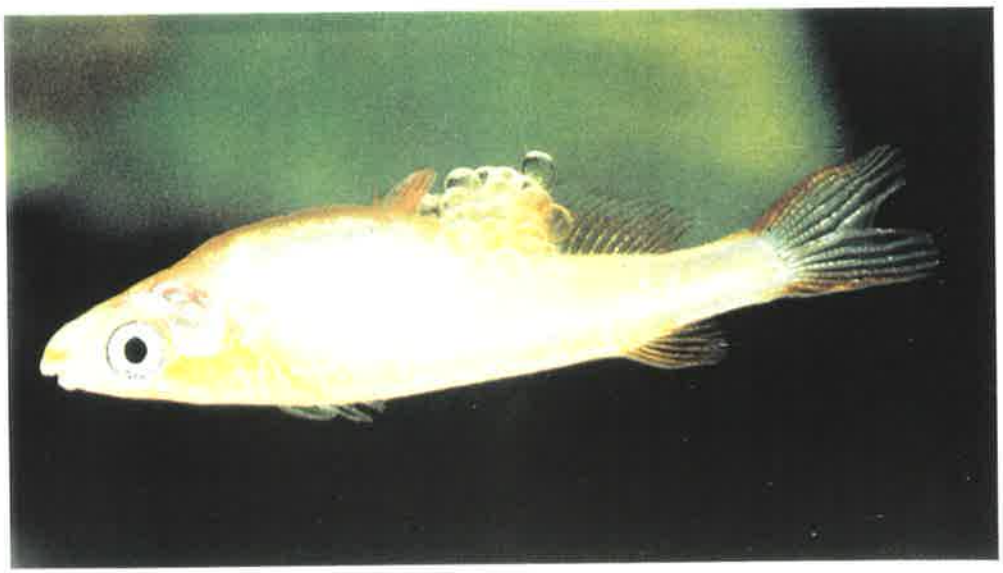
Name:	Zebrafish *Danio rerio*, belonging to the family Cyprinidae
Habitat:	Rivers of south-east India and Burma
Characteristics:	Produce large numbers of transparent eggs at regular intervals that hatch in 72-96h at 26°C.

Name:	Medaka *Oryzias latipes* belonging to the family Cyprinodontidae
Habitat:	Rice paddies of south-east Asia, hence also called Japanese rice fish
Characteristics:	Exhibits a unique reproductive behaviour with the eggs being fertilized internally and then being released outside the body. Also the females ovulate at regular intervals once ovulation begins

Name:	Leopard danio *D. frankei*, also belonging to the family Cyprinidae
Habitat:	Similar to zebrafish
Characteristics:	Related to zebrafish but a distinctly different species, adult body pigmentation consists of pigmented spots as opposed to the striped pattern displayed by the zebrafish

Plate 1.1 Species of fish used for this study





CHAPTER 1
LITERATURE REVIEW

1.1 INTRODUCTION

With the growing demands for improvement in efficiency, the aquaculture industry is increasingly looking to biotechnology for answers. Various genetic technologies are emerging ranging from transgenesis to ploidy manipulation, to enhance growth efficiency and control reproduction, which are of potentially great interest to producers. With a few notable exceptions (Streisinger *et al.*, 1981; Ozato *et al.*, 1986) most of the early development has occurred through studies on the aquaculture species of interest (Chourrout, 1980; 1982a; 1982b; Chourrout *et al.*, 1980; 1982; 1986a; Maclean and Talwar, 1984; Maclean, 1987; Thorgaard *et al.*, 1981; 1982) as there had been no generally accepted laboratory fish model on which to found and test new concepts. This contrasts with the extensive use of laboratory rodents in the development of breeding, nutritional and production techniques for farm animals. Apart from the more obvious draw backs of working with commercial fish species directly, most notably the time constraints imposed by the long generation time, this trend has had the more insidious consequence of limiting the cross pollination of ideas which occurs when researchers share a common experimental tool and provide the critical interactions which are crucial to stimulate basic research in piscines. This trend has also minimised the interest of researchers in several emergent, potentially excellent piscine models for genetic and embryological uses (Laale *et al.*, 1968; Laale 1977; Ozato *et al.*, 1989; Barinaga, 1990; Little, 1993; Helde *et al.*, 1994).

Preliminary investigations have shown the malleability of the fish genome and open up numerous possibilities for the future of genetic manipulation of fish both for commercial ventures and as research tools. The need for a convenient, fecund and cost-efficient model fish species for investigative research, has been

identified and a number of aquarium species proposed as providing potential answers to this problem (Brenner *et al.*, 1993; Morell, 1993; Kahn, 1994; Ozato and Wakamatsu, 1995). This thesis is aimed at establishing a piscine model that can be utilised in a program of research aimed at applying modern methods of genetic manipulations to aquaculture. As a result of preliminary work two species have been targeted, they are *Danio rerio* (zebrafish) and *Oryzias latipes* (medaka). The study had the aim of identifying the limitations, both biological and technical, and ascertain means of overcoming or circumventing them.

The zebrafish has in recent years been attracting increasing interest from a number of disciplines namely carcinogenicity testing, mutagenesis, transgenesis, lineage, developmental and gynogenesis studies (Khudoley, 1984; Walker and Streisinger, 1983; Mullins *et al.*, 1994; Stuart *et al.*, 1990; Patil *et al.*, 1994; Kimmel and Warga, 1987; 1988; Postlethwait *et al.*, 1994; Streisinger *et al.*, 1981). The medaka exhibits an unusual reproductive behaviour in that it is one of the few fish that displays a distinct cycle of ovulation, which has earmarked it for *in vitro* maturation (IVM) research for a number of years, and this knowledge has been utilised to develop a novel approach to transgenesis (Ozato *et al.*, 1986). The studies reported in this thesis demonstrate suitability of the targeted species as models for biotechniques of interest to the researcher and aquaculturist, including transgenesis, chromosome set manipulation, and production of chimeras. Finally the allied technologies of fish breeding, artificial insemination (AI) and larval rearing have been looked at and means of optimisation have been examined.

1.2 LITERATURE REVIEW

This review has been presented in four sections. The first examines the application of transgenesis to fish species and the various strategies employed in introducing genes into fish embryos. The next examines the relatively new

and under-explored area of embryonic stem cell isolation from fish and the implication of this to production of chimeras and ploidy-mosaic chimeras in fish. The third section deals with chromosomal manipulation or manipulation of the genome as a whole, and covers ploidy manipulation, production of androgenetic and gynogenetic diploid fish, and sex inversion. The last section relates to the range of technologies allied to fish rearing and maintenance that is imperative to any successful biotechnology program.

1.3 SINGLE GENE MANIPULATIONS

The term "*transgenesis*" was coined by Gordon and Ruddle, (1981), to describe a technical process enabling transfer of inheritable functional genes between organisms irrespective of species barriers. In recent times its sense has been expanded to include a range of techniques which allow manipulation of resident genes *in situ* (Seamark, 1989).

The introduction, expression and germ line transmission of novel genes has been successfully demonstrated in a number of invertebrates, including *C. elegans* (Stinchcomb *et al.*, 1985), *Drosophila* (Spradling and Rubin, 1982), and sea urchins (McMohan *et al.*, 1984; 1985) and lower vertebrates, frogs (Bendig, 1981).

Gene transfer was first reported in mammals by Gordon *et al.*, (1980). A major advance occurred 2 years later when Palmiter *et al.*, (1982) microinjected a gene construct, containing promoter elements to regulate the functioning of the transgene, into the pronucleus of fertilized one-cell mouse embryos. A proportion of the progeny resulting from the transfer of these embryos to the reproductive tract of surrogate mice showed integration of the transgene into the genome, with some expressing the transgene and subsequently passing it *via* their germline to their offspring. Following the success of transgenesis in

mice the technology was transferred to a number of mammalian species (Hammer *et al.*, 1985; Brem *et al.*, 1986; Pursel *et al.*, 1987; Vize *et al.*, 1988; Rexroad *et al.*, 1989; Murray *et al.*, 1989). The potential of the application of this technology to fish is huge both from the scientific and commercial view point.

1.3.1 Transgenic fish — The Pros and Cons

Transgenesis in fish was initially investigated independently by Zhu *et al.*, (1985), Chourrout *et al.*, (1986a) and Ozato *et al.*, (1986) and in some species is now developed to a point where transgenic fish are being considered for commercial release.

It was quickly recognised that from the point of view of application of transgenesis, fish varied from mammals in fundamental ways which were both advantageous and disadvantageous. Fertilisation in fish is generally external, the exceptions being the live bearing Poecillids, Xiphophorids and the Japanese rice fish *Oryzias latipes*. Thus the advantage is that eggs and milt (semen) are easily obtained, facilitating artificial insemination (AI). Relative to mammalian eggs, fish eggs are massive (> 1 mm), and this coupled with high fecundity characteristics of most fish, greatly simplifies manipulation of fish embryos. Incubation of fertilized eggs is also simple as it requires no special media or gaseous environment and their development can be very rapid, especially in warm water species such as zebrafish, and Siamese fighting fish (Laale, 1977; Kavumpurath and Pandian, 1992b). There are certain exceptions such as rainbow trout and medaka, where development to hatching may take 10 to 20 days. Most importantly no further manipulation of the embryos is required, such as re-introduction into the reproductive tract of a recipient female, as required with mammalian embryos. When attempting to apply mammalian techniques for introducing DNA *via* microinjection, several disadvantages became obvious. One disadvantage of fish embryos (also insect and amphibian)

compared with the mammal is the difficulty of visualising the relatively small zygote nucleus (pronucleus) in the voluminous cytoplasm, which is targeted when microinjecting transgenes into mammalian embryos. In the fish this necessitates microinjection of the transgene in the peri-nuclear cytoplasm (Maclean *et al.*, 1987) which reduces considerably the chances of integration into the genome although it can be compensated for to some extent by injection of larger volumes, and hence, copy numbers of the gene constructs (Zhu *et al.*, 1985; Ozato *et al.*, 1986; Stuart *et al.*, 1988; 1990). An unexpected discovery in some fish species (Stuart *et al.*, 1988), is that foreign DNA when injected into the egg cytoplasm, undergoes hitherto unexplained extra-chromosomal replication during early development, hindering the early detection of transgenics. This phenomenon has been observed in *C. elegans* (Stinchcomb *et al.*, 1985), sea urchin (McMohan *et al.*, 1985), and amphibian (Bendig, 1981; Etkin and Pearman, 1987) and only very rarely in mice (Brinster *et al.*, 1985).

The large size and independence of the embryo does however improve the survival of microinjected embryos, with survival rates of >70% being common (Chourrout *et al.*, 1986a; Brem *et al.*, 1988; Inoue *et al.*, 1989). Another technical problem relates to the difficulty of microinjection caused by the tough nature of the protective chorion or egg shell.

1.3.2 Gene Transfer Techniques Investigated

1.3.2.1 Non piscean species

A number of approaches have been investigated to transfer transgenes into the genome of animals, with varying degrees of success.

Pronuclear Microinjection

Microinjection of transgene constructs into the male pronucleus (Palmiter *et al.*, 1982) is still the most widely used technique for producing transgenic animals and has proven useful for both commercial and research purposes.

Embryonic Stem Cells

The emerging procedures of choice are based on Embryonic Stem (ES) cells (reviewed in section 1.4.1). Typically, ES cells are pluri-, if not toti-potent cells derived from the inner cell mass of blastocyst stage embryos maintained under *in vitro* conditions allowing their continuous proliferation without differentiation (Evans and Kaufman, 1981). Thus, through ES cells, transgenesis is no longer restricted to effects achievable through simple insertion of a dominant gene to a random site within the genome, as it is in the case of microinjection, but it is extended to allow manipulation (mutation) of resident genes *in situ* (Robertson, 1991) and the insertion of transgenes into specific sites through homologous recombination (Capecchi, 1989).

Sperm Mediated Transfer

Another method that promised much early on but has yet to pay dividends was the report by Lavitrano *et al.*, (1989), that mouse sperm, when incubated in the presence of high concentrations of DNA, were capable of picking up and transferring the DNA to the oocytes they fertilise. The use of sperm cells to transfer viral DNA into eggs was first described by Brackett *et al.*, (1971). Despite the phenomenon being verified for a number of species including sea urchin (Arezzo, 1989), pigs (Gandolfi *et al.*, 1989), bull, pig, ram, goat, rooster, mouse and carp (Castro *et al.*, 1990), cattle, blowfly and honey bee (Atkinson *et al.*, 1991), no germline transgenic animals have resulted. The failure of the technique to be successfully repeated in other laboratories (Barinaga, 1989; Brinster *et al.*, 1989b; Hochi *et al.*, 1990) continue to raise doubts about its potential. No details are available concerning recent claims of sperm mediated

production of transgenic sheep in China (Gu Wenyi, per comm). Further work from the original laboratory (Lavitrano *et al.*, 1992) has reopened the debate, especially as they report that long fragments of DNA (>7 kb), appear to be absorbed by the sperm preferentially over shorter strands (150-750 bp), and not only DNA, but acidic proteins are also internalised by spermatozoa, and poly-L-lysine, a poly-cation, appear to favour the uptake. They (Francolini *et al.*, 1993) have further demonstrated, using ultrastructural autoradiography, that the exogenous DNA is internalised into the nucleus. Other variations of sperm mediated transfer attempted are treatment of the sperm with liposomes (Bachiller *et al.*, 1991) and injection of DNA-liposome complexes into mouse testes (Ogawa *et al.*, 1995) or electroporation of the sperm (Gagne *et al.*, 1991; Mueller *et al.*, 1991) to facilitate their uptake of DNA.

Alternative Gene Transfer Methods

A number of alternate gene transfer methods have been investigated; such as pricking blastocysts in the presence of plasmids to stimulate up take of the DNA by the inner cell mass (ICM) of the blastocyst (Sato *et al.*, 1993), intravenous injection of expression plasmid-cationic liposome complexes into adult mice which was found to transfect all tissues with the transgene active for up to 9 weeks (Wolff *et al.*, 1990; Zhu *et al.*, 1993). Biological ballistics or "*biolistics*" is a relatively new animal cell transfection method, originally developed for plants (Sanford *et al.*, 1987), that literally shoots high velocity microprojectiles, carrying DNA, into cells. It has been used to successfully transfect plants, organelles, microbes, fish eggs, drosophila embryos, mammalian cells and mouse tissue *in situ* (reviewed by Pecorino and Lo, 1992), and shows promise for the future if it results in germ line transformation.

1.3.2.2 Pisceans

MICROINJECTION

In fish also, microinjection (into the peri-nuclear region) is the most widely used technique. The problem of the tough chorion has been tackled in different ways by various investigators. Dechorionation by digestion and/or mechanical removal has been successfully used for species such as goldfish and loach, *Misgurnus anguillicaudatus* (Zhu *et al.*, 1985) and zebrafish (Stuart *et al.*, 1988), where development to hatching is very rapid. Rainbow trout embryos can be successfully microinjected shortly after fertilisation, prior to the hardening of the chorion (Maclean and Talwar, 1984), or by the exacting approach employed by Chourrout *et al.*, (1986a), where a hole is drilled in the chorion of each embryo and then they are injected through it (McEvoy *et al.*, 1988; 1992). Alternatively, glutathione can be used to prevent hardening of the chorion (Oshiro *et al.*, 1989; Inoue *et al.*, 1991). Microinjection of eggs through their micropyle prior to activation has been successful for salmon (Shears *et al.*, 1991) and tilapia (Brem *et al.*, 1988).

A novel approach used by one laboratory was to inject the transgene into the germinal vesicle (GV) of the immature medaka oocyte and then to induce the oocyte to undergo final maturation and ovulation, *in vitro* before effecting fertilisation by artificial insemination (Ozato *et al.*, 1986). A lack of knowledge of *in vitro* maturation (IVM) for most species limits the use of this unique approach.

ELECTROPORATION OF EMBRYOS

Electroporation of embryos soon after fertilisation, has been successfully used to transfer genes into medaka (Inoue *et al.*, 1990) and zebrafish (Buono and Linser, 1991; Powers *et al.*, 1992) embryos in preliminary studies, but has been ineffective when used for goldfish (Hallerman *et al.*, 1989).

SPERM MEDIATED TRANSFER

The use of sperm as vectors to facilitate the uptake and transfer of foreign DNA into the embryos they fertilise has attracted considerable attention, as it would be ideally suited to transgenesis in fish, if it works efficiently. Khoo *et al.*, (1992), produced transgenic zebrafish by sperm mediated transfer (37.5% or 3/8), however, they did not observe expression despite the fact that the construct used was the same one that Stuart *et al.*, (1990), microinjected to successfully induce expression and germline transmission in the same fish species. Chourrout *et al.*, (1992), found sperm mediated transgenesis to be ineffective for producing transgenic rainbow trout.

1.3.3 Gene Constructs Investigated

The technology of gene cloning has made the production of transgenic animals a reality. Plasmids of genomic or complementary DNA (cDNA) may be cloned to make the constructs for transgenesis. cDNA spliced to a promoter is most often used, as the increase in size of the transgene as a result of using genomic DNA can pose technical problems during the injection procedure. Circular (Oshiro *et al.*, 1989), linear (Maclean *et al.*, 1987; 1989; Stuart *et al.*, 1988) or supercoiled (Stuart *et al.*, 1988; Ozato *et al.*, 1989a) gene constructs have been used successfully for fish transgenesis studies. Linear plasmids are reported to induce higher rates of transformation (60-70% as opposed to 10-30% for circular plasmids) in frogs (Etkin *et al.*, 1984) and (75% and 40% respectively) in fish (Chourrout *et al.*, 1986a). There are only a few reports of the use of supercoiled plasmids with conflicting results in zebrafish with Stuart *et al.*, (1990), reporting integration, expression and germline transmission but Bayer, (1992), having limited success.

1.3.3.1 Promoters

Increasing the expression of the genes is effected by attaching them to a powerful universal promoter from another gene (usually a house keeping gene), such as the mouse Metallothionein promoter (Brem *et al.*, 1988; Alok and Khillan, 1989; Chen *et al.*, 1990; Hernandez *et al.*, 1991; Rahman and Maclean, 1992), the long terminal repeat promoter (LTR) of the Rous Sarcoma Virus (Zhang *et al.*, 1990; Stuart *et al.*, 1990; Tamiya *et al.*, 1990; Hallerman *et al.*, 1990; Inoue *et al.*, 1991; Gross *et al.*, 1992; Phillips *et al.*, 1992), or the SV40 viral promoter (Chourrout *et al.*, 1986a; Stuart *et al.*, 1990; Khoo *et al.*, 1992). In a few studies other promoters have been utilised, (Inoue *et al.*, 1991) but they remain exceptions. Comparative efficiency of promoters has not been widely examined but one study on zebrafish (Gibbs *et al.*, 1991), list them in ascending order of CMV>RSV>SV2>SV0. Studies with fish cell lines estimate RSV and CMV to have similar efficiencies (Bearzotti, 1992).

1.3.3.2 Genes

All the initial work was done using genes isolated from mammals, and genes which show expression in the entire animal, such as growth hormone genes, were preferred over tissue specific ones (Zhu *et al.*, 1985; Chourrout *et al.*, 1986a; Brem *et al.*, 1988; Alok and Khillan, 1989; Male *et al.*, 1989; Schneider *et al.*, 1989; Zhang *et al.*, 1990; Chen *et al.*, 1990; Hernandez *et al.*, 1991; see also review by Chen and Powers, 1990), with some exceptions such as the chicken *delta-crystallin* gene in medaka (Ozato *et al.*, 1986; Inoue *et al.*, 1989b) and the flounder *anti freeze protein* gene in salmon (Davies *et al.*, 1989). Construction of gene libraries for rainbow trout (States *et al.*, 1982), Atlantic salmon and grass carp (cited in Maclean *et al.*, 1987), and chum salmon (Sekine *et al.*, 1985) have opened the way to the use of piscine cDNA for genetic studies. An interesting use of piscine genes, is the attempt to use the anti-freeze protein gene, isolated from artic flounder, to impart resistance against freezing temperatures, to Atlantic salmon (Shears *et al.*, 1991).

Reporter genes, such as the genes coding for β-galactosidase (*lacZ*), Chloramphenicol Acetyl Transferase (*cat*), and Luciferase (*luc*), have often been used to optimise transfer techniques as they are readily available and have routine assays that are easily adapted to suit different applications (McEvoy *et al.*, 1988; Hallerman *et al.*, 1989; Tamiya *et al.*, 1989; 1990; Stuart *et al.*, 1990; Inoue *et al.*, 1991; Lin *et al.*, 1994; Patil *et al.*, 1994).

Of the three reporter genes mentioned, the *cat* and *lacZ* genes have a serious limitation with regard to fish transgenesis, in that the gut bacteria tend to confuse the results with background staining appearing even in the controls. The firefly luciferase (*luc*) gene shows a great deal of promise, highlighted by the production of transgenic "*glowing*" tobacco plants (Ow *et al.*, 1986). Using a luminometer, which is the standard assay equipment for luciferase, the sensitivity of the LUC assay is 30-1,000 fold more than CAT (de Wet *et al.*, 1987). Since the LUC assay was optimised for use as a reporter gene in mammalian cell lines (Brasier *et al.*, 1989), and scintillation counters were effectively used for the assay (Nguyen *et al.*, 1988), it is the reporter gene of choice. Its utility was demonstrated when it was used to produce transgenic mice (Di Lella *et al.*, 1988). It has only more recently been used in fish transgenesis studies (Tamiya *et al.*, 1990; Gibbs *et al.*, 1991).

1.3.3.3 Future Applications

In the future, transfer of antisense DNA sequences or genes that impart resistance against bacterial, fungal, or viral diseases, such as Infectious Haematopoietic Necrosis Virus (IHNV), Bacterial Kidney Disease (BKD) and furunculosis could be of great importance to aquaculture operations (Maclean, 1989; Chen and Powers, 1990). Other applications could be to use transgenic fish as potential expression systems for pharmaceutical products (Maclean and Penman, 1990), or to "*imprint*" fish with marker DNA sequences in order to facilitate population studies in the wild (Maclean, 1989).

1.3.4 Assessment of Transgenics

To fulfil their potential transgenes must be integrated in the genome, produce the proteins they code for and finally be transferred *via* the germ line to subsequent generations (Maclean *et al.*, 1987), and although studies that combine all three steps in fish are rare (Ozato *et al.*, 1989), successes have been reported for carp and loach (Powers *et al.*, 1991), and zebrafish (Stuart *et al.*, 1988; 1990). Studies where one or two of the above mentioned steps have been achieved are numerous (see reviews by Maclean *et al.*, 1987; Ozato *et al.*, 1989; Chen and Powers, 1990; Houdebine and Chourrout, 1991).

1.3.4.1 Mosaicism

Mosaic integration of transgenes (or integration after the embryos has undergone a few rounds of cell cleavage) in transgenic founder animals appears to be extremely common in fish transgenesis studies (Houdebine and Chourrout, 1991) and has been observed by DNA-DNA *in situ* hybridization (Ozato *et al.*, 1986), DNA estimation and transmission to offspring studies (Stuart *et al.*, 1988; Schneider *et al.*, 1989; Penman *et al.*, 1991; Culp *et al.*, 1991), or by histochemical studies (Ozato *et al.*, 1986; 1989; Inoue *et al.*, 1989b; 1991). The rapid cleavage rates of fish could exacerbate mosaicism, however, though it is undesirable it may not be a grave problem as transgenic individuals of subsequent generations are usually fully hemizygous for the transgene (Stuart *et al.*, 1990;). The possible fates of sequences of foreign DNA injected into fish embryos has been reviewed by Maclean *et al.*, (1987).

1.3.4.2 Integration

In fish Dot blot and Southern blot DNA hybridization has been the most commonly used technique to verify integration of the transgene (Ozato *et al.*, 1986; Brem *et al.*, 1988; Stuart *et al.*, 1988, 1990; Oshiro *et al.*, 1989; Schneider *et al.*, 1989; Inoue *et al.*, 1990; Penman *et al.*, 1990; Buono and Linser, 1991; Khoo *et*

al., 1992) with PCR being used in occasional studies (Male *et al.*, 1989; Oshiro *et al.*, 1989; Culp *et al.*, 1991; Patil *et al.*, 1994).

1.3.4.3 Expression

Once the integration of the transgene into the founder F0 generation has been verified the next point of interest is whether the transferred gene has survived in a functional state and can synthesise the protein it codes for. All the above mentioned reporter genes used have well documented convenient assays to determine their levels of expression, also some of the other constructs like the ones containing GH genes can induce phenotypic effects which facilitate identification of expressing transgenics (Maclean *et al.*, 1987; Zhang *et al.*, 1990; Alok and Khillan, 1989). Tamiya *et al.*, (1990), using the luciferase reporter gene have devised an extremely sensitive system that assays living embryos.

1.3.4.4 Germline transmission

The third and possibly most salient point in transgenesis studies is the ability of a transformed individual to pass the acquired transgene onto its progeny in a functional form. Germline studies are usually performed by breeding transgenic founders with non-transgenic individuals to determine the fraction of the resultant progeny that retains the transgene (Culp *et al.*, 1991). Attempts at using gametes produced by founder individuals for determining the presence of the transgene have proved inconclusive (Penman *et al.*, 1991).

1.4 PARTIAL GENOME MANIPULATION

The field of production of fish chimeras is relatively unexplored as the manipulation of embryos requires sophisticated equipment and operator skills, also the back-up technology of culture of embryonic stem cells in fish is only at an early stage.

1.4.1 Embryonic Stem cells

In the early 1980s Evans and Kaufman, (1981) and Martin, (1981) demonstrated the possibility of establishing pluripotential cells derived from the inner cell mass (ICM) of mouse blastocysts in culture, moreover these cells, called embryonic stem (ES) cells, are capable of resuming their full developmental potential when they are reintroduced into a recipient mouse blastocyst and have the ability to contribute efficiently to all the embryonic tissues of the resulting embryo including the germline.

ES cells have been shown to be able to tolerate manipulation *in vitro* without losing their capacity to generate germline chimeras (Robertson *et al.*, 1986). Based on the coat-colour alleles of the ES cells being distinguishable from the recipient blastocyst the resultant chimeric mouse will display coat-colour mosaicism. The contribution of the ES cells to the germline of the chimera can be evaluated by breeding to a suitable mouse.

The availability of mouse ES cells raises the possibility that mice carrying a specifically modified gene can be obtained by manipulating the target gene in the ES cells *in vitro* by techniques such as homologous recombination (HR) (see review on Gene Targeting by Capecchi, 1989) and introducing the resultant cells into appropriate blastocysts.

Unlike mammals where stem cell technology for mice has been around for a while, putative fish stem cells have only recently been isolated from medaka (Ozato *et al.*, 1995), but initial attempts to use them to create chimeras have not been conclusive. Mitogenic activity in trout embryo extract that maintains and promotes growth of primary salmonid embryo cell cultures has been reported (Collodi *et al.*, 1990; 1991), and promises a break-through in the isolation of fish stem cells in this species. Similar work on zebrafish (Collodi *et al.*, 1992) has demonstrated the ability of zebrafish cell cultures to be transfected with

procedures utilised in mammalian systems and to express these constructs in a stable manner. However, the high rates of transgenesis achieved for some species of fish may permit gene targeting directed at embryos, thereby making the need for ES cells superfluous (Lin *et al.*, 1992).

1.4.2 Aggregation Chimeras

The experimental production of chimeras has been an extremely useful tool for investigating fundamental aspects of early embryonic development and differentiation in mammals (McLaren, 1976) or as a method of introducing foreign genes into mice by incorporation of transgenic ES cells into blastocysts (Robertson *et al.*, 1986). In domestic animals such as pigs (Kashiwazaki *et al.*, 1992), cattle (Picard *et al.*, 1990), sheep and goats (Polzin *et al.*, 1987; Roth *et al.*, 1989) where ES cells have yet to be isolated, chimeras have been produced by aggregation or injection of ICM cells into blastocysts.

Intra-specific chimeras have been produced by injecting isolated blastomeres from blastula stage pigmented embryos into colour-mutant embryos at the same stage for zebrafish (Lin *et al.*, 1992), and medaka (Ozato *et al.*, 1995), to determine whether the recipient embryos accept or reject the foreign cells in the first place, and further whether these introduced cells contribute to the phenotype and the genotype of the resulting embryo. These studies demonstrated the ability of disaggregated blastomeres isolated from donor embryos to combine with recipient (colour-mutant) embryos of the same species successfully and contribute to the phenotype and genotype of the resulting chimera. No other species of fish has been investigated for the purpose of generating chimeras, although successful nuclear transplants in fish have been reported (Gasaruan *et al.*, 1979; Shaoyi, 1990).

1.4.3 Hybridization in fish

The feasibility of creating chimeric fish between two different species has not as yet been investigated. However, hybridization in fish has been extensively studied (Purdom, 1972; Mekeyeva, 1975; Stanley, 1976a; Magee and Philipp, 1982; Ueda *et al.*, 1984; Johnson and Wright, 1986) and is common between closely related species of fish; hybrids thus produced often exhibit an intermediate phenotype between the two parental phenotypes (Ueda *et al.*, 1984; Johnson and Wright, 1986; Kavumpurath and Pandian, 1992a). Diploid hybrids and gynogenetic diploids have been reported in grass carp, *Ctenopharyngodon idella* (Sutton *et al.*, 1981). Mekeyeva, (1975) observed viable gynogens and inviable hybrids resulting from the cross between *Hypopthalmichthys molitrix*X*Cyprinus carpio*. The crosses between *Danio rerio* X *D. frankei* and their reciprocal cross failed to produce any gynogenetic diploids, but the hybrids displayed a colour pattern intermediate between the two parental species suggesting action of co-dominant alleles (Kavumpurath and Pandian, 1992a).

1.4.4 Ploidy Mosaic chimeras

A novel application of chimeras would be as a means of producing ploidy mosaic (4N-2N) chimeras, if tetraploid fish prove to be inviable. For this to be attempted, early identification of tetraploid embryos would be a necessity.

In mammals 4N embryos survive to the hatched blastocyst stage and can implant at a high frequency which is longer than haploid or triploid individuals (Snow *et al.*, 1975, 1976; Tarakowski *et al.*, 1977) and they have been used as recipient blastocysts for stem cell injection, to increase the frequency of integration of injected ES cells in the resulting chimera (Nagy *et al.*, 1990). No one has reported attempts at such manipulations in fish as early identification of tetraploid fish embryos requires the embryos to be sacrificed.

1.5 ENTIRE GENOME MANIPULATIONS

Fish as opposed to mammals have proved to be surprisingly tolerant of alterations to their ploidy status and naturally occurring triploid fish have been identified (Gold and Avise, 1976; Thorgaard and Gall, 1979). The first reports of induction of polyploidy in fish go back almost four decades (Swarup, 1956; 1959a). This was achieved by blocking early cell division events of embryos at varying times and by various means to produce tetraploid or triploid individuals. The latter are of special interest to researchers working in the area of transgenesis due to their sterility, which results from the odd number of chromosomes being unable to pair at meiosis leading to the formation of aneuploid gametes, an outcome of particular importance if the benefits of transgenesis are to be realised. By contrast the interest in tetraploids is that if they are viable and fertile, they can be used in the production of entirely sterile triploid populations as was demonstrated in amphibians (Jaylet, 1972). Androgenesis (all paternal inheritance) and Gynogenesis (all maternal inheritance) are also made possible in the fish by the ability to inactivate the chromosomes of either the egg or the sperm prior to fertilisation then double the chromosome number of the resulting haploid embryo, to recover its diploid status. The capacity to cause sex inversion in individuals through the use of androgens and estrogens, is a key factor to maximising the benefits gained by such chromosome set manipulations (Nagy *et al.*, 1981; Nagy and Csanyi, 1984; Shelton, 1986).

(A) CHROMOSOME SET MANIPULATIONS

Polyploid ancestry is an accepted fact in a number of fish species, and some groups such as the catostomids and the salmonids clearly reflect a tetraploid event in the ancestral form (reviewed by Schultz, 1980). Examples of apparently recent polyploids are poeciliid fishes, some loaches and the unisexual goldfish, demonstrating that new polyploids can sometimes be viable

and fecund. The main thrust of polyploidy induction is directed towards the production of sterile triploids as an all female, triploid population would be the most effective sex control mechanism. However, induced polyploidy can also result in increased mortality and infertility due directly to the effects of chromosome doubling or caused by the treatment used to induce polyploidy.

1.5.1 Triploidy

Triploidy has been successfully induced in a growing number of fish species *eg.*, the three spined stickleback, plaice, tilapia, rainbow trout, zebrafish, grass carp, pacific salmon, coho salmon (Swarup, 1959a; 1959b; Purdom, 1972; Valenti, 1975; Chourrout, 1980; 1984; Streisinger *et al.*, 1981; Utter *et al.*, 1983; Johnson *et al.*, 1986; Van Eenennaam *et al.*, 1990) and this supports the expectation of viability of triploids, especially as the incidence of spontaneous triploids were reported in a few fish species *e.g.*, rainbow trout, the California roach, (Cuellar and Uyeno, 1972; Thorgaard and Gall, 1979; Gold and Avise, 1976). This success and good viability seen is similar to that obtained for triploid amphibians (Frankhauser, 1945). This is in total contrast to experience with triploid mammals which are unable to survive much more than pre-implantation stages of cellular development (Niebuhr, 1974). The enhanced growth and sterility of some triploid fish strains are of considerable interest to the aquaculturist (Wolters *et al.*, 1991; Mclean *et al.*, 1991; Sugama *et al.*, 1992; Recoubratsky *et al.*, 1992).

1.5.1.1 Induction of Triploidy

Triploidy can by induced "***de novo***" in one of two possible ways, either by retention of the first polar body or retention of the second polar body (Purdom, 1970). The first option is impractical as the first polar body has degenerated long before fertilisation. Therefore the retention of the second polar body is the accepted route of intervention, and this is believed to be the event that leads to

the naturally occurring triploids reported above. Second polar body retention can be induced by temperature, pressure, or chemical treatment of the fertilized egg and the commercial and research applications of triploidization are well documented (Thorgaard and Gall, 1979; Thorgaard, 1983; Utter *et al.*, 1983; Chourrout *et al.*, 1986b; Don and Avtalion, 1986; Thorgaard, 1986; Arai and Wilkins, 1987; Kavumpurath and Pandian, 1990; Varadaraj and Pandian, 1990). Suppression of polar body extrusion can also occur in inter-specific crosses, resulting in triploid hybrids. Vasil'ev *et al.*, (1975) for example have reported on hybrids from crosses between carp and other Cyprinidae, producing triploid offspring. The hybrid between female grass carp and male bighead carp (*Aristichthys nobilis*) yield a high frequency of triploid offspring (Marian and Krasznai, 1978; Beck *et al.*, 1980).

Temperature Shock

Temperature treatments have been widely used as a means of suppressing second polar body extrusion in fish. Both heat shock (Swarup, 1956; Chourrout, 1980; Thorgaard *et al.*, 1981) as well as cold shock (Swarup, 1959a; Purdom, 1969; 1972; Valenti, 1975; Ojima and Makino, 1978; Nagy *et al.*, 1978; Lemoine and Smith, 1980; Chourrout, 1980; Wolters *et al.*, 1981; Refstie *et al.*, 1982) treatments have been used efficiently. The physical parameters such as timing, duration and temperature must be determined for each species. The practicality, convenience and logistics of temperature shock thus make it a method of choice from an aquaculturists' point of view. Though for some species, especially the salmonids, there may be limitations (Chourrout, 1980).

Pressure Shock

Hydrostatic pressure shock has been successfully used to induce second polar body retention in zebrafish and rainbow trout (Streisinger *et al.*, 1981; Yamazaki, 1983; Chourrout *et al.*, 1982; Chourrout, 1982a; 1984). The drawback of this method is the specialised equipment required, including pressure cells

and hydraulic presses, and the limitations exerted by the size of the pressure containers. Thorgaard, (1983) however, advises against discounting this method as fish could show similarities with salamanders, a genus where pressure generated triploids display much greater survival than their heat shock derived counterparts. Also there seem to be, as yet undetermined, factors that favour different treatments for different species.

CHEMICAL SHOCK

Chemicals which have been used successfully to block polar body extrusion in animals include Cytochalasin B. (Allen and Stanley, 1979; Refstie *et al.*, 1977; Refstie, 1981). Exposure of fertilised eggs to this chemical has been reported to produce mosaic polyploid-diploid Atlantic salmon embryos, and triploid Manila clam, *Tapes semidecussatus* embryos (Beaumont & Contaris, 1988), and exposure of embryos to colchicine have been found to cause similar mosaicism in brook trout (Smith and Lemoine, 1979). The potential use of chemicals like polyethylene glycol which have induced polyploidy in mouse embryos (Eglitis, 1980), could also be of interest. In general, the major drawback of using chemical treatments for fish is the fact that all the chemical agents mentioned are extremely toxic and pollution could be a consideration, also they cannot be precisely controlled especially for species that display an extremely narrow, defined window for such manipulations. An additional disadvantage is the restriction on the number of eggs that can be treated.

1.5.1.2 Survival and Viability of Triploids

Triploids have been studied in a number of species mentioned above and they have been found to have normal viability in a number of species such as, tilapia, zebrafish, tench (Don & Avtalion, 1986; Kavumpurath & Pandian, 1990; Flajshans *et al.*, 1993a; 1993b). The exceptions are the salmonids — the rainbow trout (Thorgaard *et al.*, 1982; Lincoln and Scott, 1983; 1984; Grey *et al.*, 1993) and coho salmon (Utter *et al.*, 1983) — suggesting that triploid individuals in these

species may be sub-vital. Work by Chourrout *et al.*, (1986b), and Myers & Hershberger, (1991), has shown that triploid rainbow trout derived from a 4N-2N mating (second generation triploids) exhibit a much better viability than first generation (*de novo*) triploids, suggesting an effect related to the treatment rather than the polyploid state. Sexual development of triploids has been studied for a number of species *e.g.*, zebrafish, tilapia, grass carp, Pacific salmon, coho salmon (Kavumpurath and Pandian, 1990; Varadaraj and Pandian, 1990; Van Eenennaam *et al.*, 1990; Utter *et al.*, 1983; Jhonson *et al.*, 1986). Complete blockage of female gonadal development has been observed in all species studied. In fact, all-female triploid production of rainbow trout is already being used commercially (Bye & Lincoln, 1986).

A further spin off of this technology was the observation that some triploids showed an apparent diversion of gonadal growth to somatic growth. Female triploid tilapia grow 14% faster than monosex (sex-reversed) males and 23% faster than male triploids (Vardaraj and Pandian, 1990). Triploid tench *Tinca tinca* produced by either the cold shock or hydrostatic pressure route produced high levels (>80%) of viable 3N fry, with the females showing an increase in live weight of 13.6% with the Gonado-Somatic Index (GSI) diminishing to 25% of control values. 3N males had an even greater increase in live weight (27%) with their GSI reaching 60% of control values (Flajshans *et al.*, 1993b).

In salmonids the GSI of 3N coho salmon females was 11.8% and 3N males was 35.7% of controls respectively, at 30 months of age (Jhonson *et al.*, 1986). Tetraploid derived 3N rainbow trout showed a significant increase in weight over controls early, but by 120 days of culture they were similar to first generation triploids as well as controls. Other researchers have found 3N salmonids to be less vital than the controls (Utter *et al.*, 1983; Parsons *et al.*, 1986; Happe *et al.*, 1988), but they still have a useful role for aquaculture as the high mortality associated with sexual maturity, is reduced drastically (Thorgaard &

Gall, 1979). Triploid hybrids can also show an increased resistance to viruses. This is indicated by 3N rainbow trout X coho salmon hybrids which display a significant increase in Leong Type 2 virulent IHN resistance when compared with triploids of either pure species (Parsons *et al.*, 1986). Triploid male grass carp *C. idella*, despite producing abnormal aneuploid sperm can, in isolated instances, produce viable offspring (Van Eenennaam *et al.*, 1990) but milt from triploid zebrafish failed to fertilise eggs (Kavumpurath and Pandian, 1990).

1.5.2 Tetraploidy

Tetraploidy has been touted as a genuine alternative route to producing triploids at a high frequency and in a reproducible manner by mating viable tetraploids with diploids to yield triploids (Refstie *et al.*, 1977; Chourrout, 1984).

1.5.2.1 Induction of tetraploidy

Tetraploidy can be induced by blocking the first mitotic (karyokinesis), or the first cleavage division (cytokinesis), by shock treatments similar to those used for triploidy induction. The majority of success to date has been with salmonids. Tetraploid rainbow trout have been successfully produced by blocking the first mitotic division by heat shock (Thorgaard *et al.*, 1981; Chourrout, 1982) and by pressure treatment (Chourrout, 1984; Chourrout *et al.*, 1986b; Myers *et al.*, 1987; Foisil & Chourrout, 1992).

1.5.2.2 Survival and Viability of Tetraploids

SALMONIDS

In this family mortality of tetraploids have always been very high and viable survivors if any, have been few. In rainbow trout survival of both heat shock (Chourrout, 1982) and pressure shock induced tetraploids was low (20-25%) (Chourrout, 1984; Chourrout *et al.*, 1986b; Foisil & Chourrout, 1992) but some did reach maturity. None of the 2 year old female tetraploids ovulated, but 1

and 2 year old male tetraploids produced sperm that, when mated with diploid females produced very high frequencies of triploid progeny with high survival rates. The fertilising ability of the sperm from pressure induced tetraploids was low (0-97%), averaging 49% when compared with controls. Myers & Hershberger, (1991) also found that almost all of the progeny resulting from a 4N-2N cross were triploids.

To verify whether the "*double diploid*" or homogametic makeup of tetraploids due to mitotic inhibition, is the major cause of the reduced viability Chourrout *et al.*, (1986b) compared first generation tetraploids produced by pressure treatments, with second generation tetraploids produced by subjecting triploids, derived from a 4N-2N cross, to a heat shock aimed at retention of the second polar body. Survival of tetraploids produced in this way showed increased survival and growth over first generation tetraploids. The spermatocrit (part of milt occupied by the spermatozoa) of tetraploids was lower (19%) than controls (35%) and the ratio of sperm head diameters between tetraploids and diploids was 1.30 and for length was 1.20. The variation in head diameter shows a correlation with fertilisation success which indicates that narrower sperm fertilise better. They have also demonstrated that sperm from tetraploids, even though they cannot always fertilise eggs themselves can successfully prevent fertilisation by other sperm. Growth rates of polyploids at one year of age is in the descending order of second generation triploids, diploids, first generation triploids, and then second generation tetraploids, but despite being at the bottom of the list the growth of second generation tetraploids is satisfactory with an incidence of 94.5% males.

NON-SALMONIDS

Tetraploidy has been induced in non-salmonids by a combination of pressure and cold shock treatments in *Oreochromis* species (Myers, 1986a). *O. niloticus* or *O. mossambicus* when used in a homogametic cross displayed an incidence of

tetraploidy of 5.0% on average. Survival to eyed stage was highly variable ranging from 9.9-67.5%. The highest frequency (29%) of tetraploidy resulted in the male *O. niloticus*Xfemale *O. mossambicus*. Tetraploid embryos were markedly subvital, lacking the typical abundance of blood vessels usually on the yolk sac. Developmental abnormalities, particularly eye deformities, were highly pronounced and all succumbed within 7 days of hatching. Survival differences between *allo-* and *auto-* tetraploids were apparent and favoured *allo-* tetraploids, making the mating of tetraploids with tetraploids to increase heterozygosity the favourable option.

Chourrout *et al.*, (1986b), hypothesized that heat shock affects cellular mechanisms to a degree that it caused polyploidy completely or inhibited the process altogether. However, Cassani *et al.*, (1990a) observed 2N-4N mosaic embryos among their experimental group at varying frequencies resulting from both heat shock and pressure treatments. The incidence of tetraploidy in both pressure and heat shock treatment groups were high, varying from 25-100%, but no tetraploid larvae survived past 50 days post fertilisation (pf). They attributed the subvitality of tetraploids to problems associated with incomplete polyploid conversion including 2N-4N mosaics, aneuploidy and hypo-tetraploidy, and the fact that the numbers of cells present in tetraploids were substantially lower than in diploid fish.

Valenti, (1975) found several tetraploids among blue tilapia eggs treated with cold shock that were larger than controls and triploids at 14 weeks of age. Flajshans *et al.*, (1993a) also demonstrated in the tench that the survival of viable, pressure shock induced tetraploids (61.82%) is slightly better than those produced by heat shock treatments (41.46%). Reddy *et al.*, (1990) performed similar studies with the two major Indian carp species, *Labeo rohita* and *Catla catla* and were able to successfully induce viable tetraploids in *L. rohita* with both cold and heat shock at rates of 55% and 70% respectively. By contrast for

C. catla, cold shock was ineffective, but heat shock treatments resulted in 30-65% viable tetraploids being produced.

It is still early days in the study of tetraploidy especially for the commercially important non-salmonids like the cyprinids. No survival, growth and reproduction data is available as yet for this important group so it would be pure speculation as to whether tetraploid cyprinids would provide a practical means of producing triploids in a consistent and efficient manner.

1.5.3 Analysis of Polyploidy

The methods for analysis of the ploidy status of fish or embryos are as varied as the methods of chromosome set manipulations. The disadvantage with all the methods listed below is that the larvae *must be sacrificed* if an early analysis is required or the larvae have to be reared, often for months, to a size where a tissue sample may be obtained without sacrificing the animal. This has biased researchers towards an "*overkill*" situation in which they use the greatest permissible shock to maximise the rate of altered ploidy, but which conversely results in compounding any deleterious treatment effects.

1.5.3.1 Karyotyping

Chromosome counts based on metaphase chromosome spreads has been by far the most often used and reliable method (Chourrout, 1984; Chourrout *et al.*, 1986b; Don & Avtalion, 1986; Kavumpurath & Pandian, 1990), the only drawback being the time consuming and tedious nature of this method.

1.5.3.2 Erythrocyte (RBC) Nuclear Volume

Erythrocyte (RBC) nuclear volume estimation has been used to identify polyploid individuals as the volume of the nucleus can provide an idea of the ploidy status (Swarup, 1959b; Purdom, 1972; Valenti, 1975; Allen & Stanley,

1978; 1979; Don & Avtalion, 1986; Arai *et al.*, 1991). Unfortunately it does not always provide an accurate measure (Thorgaard and Gall, 1979; Wolters *et al.*, 1982).

1.5.3.3 Flow Cytometer

The estimation of the DNA content of embryos at various stages using a Flow Cytometer has also been utilised successfully (Utter *et al.*, 1983; Allen, 1983; Cassani *et al.*, 1990a).

1.5.3.4 Coulter counter

Cassani, (1990b), demonstrated the ease of use of a coulter counter in estimating the cell number of grass carp embryos as different stages of development and relating this to the ploidy status of the embryo.

1.5.3.5 Nuclear Components

The number of nucleoli in a cell can be used to estimate ploidy. Cherfas & Ilyasova, (1980a), used nucleolar counts to identify triploid hybrids of the crucian carp and common carp. Polyploidy of tench embryos were checked by quantifying Ag-stained nucleolar organizer regions (NORs) (Flajshans *et al.*, 1993a; 1993b).

1.5.3.6 Protein Electrophoresis

Protein electrophoresis has also been used to identify polyploid fish. Liu *et al.*, (1978), used muscle myogen and creatine kinase pattern differences to identify triploid and diploid ginbuna *Carassius auratus langsdorfi.* Allen & Stanley, (1983), and Magee & Philipp, (1982), have successfully used electrophoresis to screen grass carp X bighead carp hybrids for polyploidy because of differences in relative dosages of alleles between diploid and polyploids resulting from interspecific crosses.

1.5.3.7 Phenotypic Markers

Chromosome set manipulated embryos can in some instances be identified due to phenotypic markers of parental origin such as the lack of pigment or pigmentation pattern (Streisinger *et al.*, 1981).

(B) MONOSEX POPULATIONS

A population of fish of the same sex is desirable to the aquaculturist for a number of obvious reasons most important being reduction in reproductive stress and aggression as a result of courtship, fish of one sex being more desirable, or environmentally, having a monosex population of introduced fish reduces the risk of native species being competed out (Shelton, 1986; Nagy and Csanyi, 1984; Vardaraj and Pandian, 1990). The manner in which this can be brought about can be sub-divided into three areas described below.

1.5.4 Gynogenesis

If successful, this results in an all female population as a result of an all maternal inheritance, without the paternal compliment of genetic information carried by sperm, which is inactivated prior to fertilisation. Polyploidy is then induced with the aim of doubling the chromosome set of the haploid eggs allowing them to attain the normal diploid status. Populations of crucian carp and several species of Mexican poecillidae reproduce naturally by gynogenesis (reviewed by Gold, 1979; Cherfas 1981) with the sperm from males of another species effecting fertilisation and activation of the egg but not contributing genetically. Species that are not naturally gynogenetic have occasionally exhibited a similar phenomenon when inter-crossed. Such hybrid gynogenesis has been reported (Uyeno, 1972; Purdom & Lincoln, 1974; Stanley, 1976a; Cherfas, 1981), and is an interesting prospect but it may not be reliable enough for gynogenesis studies.

1.5.4.1 Sperm Inactivation

Sperm inactivation is a more reliable way to produce genetically incompetent sperm that can still activate the egg genome. In frogs Hertwig, (1911 cited in Thorgaard 1983), showed radiation to be a good means of inactivating sperm. He described the unusual phenomenon now called the "*Hertwig Effect*", whereby survival of eggs fertilized by irradiated sperm is initially inversely proportional to the radiation dose, then increases with increasing doses of radiation. The reason for this reversal of the trend with increasing doses of radiation is attributed to total destruction of the chromosomes in the sperm at high levels of radiation resulting in production of haploid embryos which have been shown to survive longer than the diploid or aneuploid embryos, which can express dominant lethal mutations induced by the radiation treatment. Despite this haploid amphibians and fish typically die early in development (Frankhauser, 1945; Purdom, 1969). Oppermann (1913 cited in Thorgaard, 1983), was the first to use radiation treatments to produce haploid fish embryos using brown trout, *Salmo trutta*.

RADIATION

A variety of radiation and chemical treatments are available to inactivate sperm chromosomes. Radiation treatments that have been successfully utilised for this purpose include X-rays (Stanley and Sneed, 1974), χ-rays usually from ^{60}Co or ^{137}Cs sources (Purdom, 1969; 1970; Nagy *et al.*, 1978; Ijiri, 1980; Chourrout *et al.*, 1980; Onozato, 1982; Refstie and Donaldson, 1982), and ultraviolet (UV) light (Nace *et al.*, 1970; Stanley, 1968;1976; 1983; Ijiri and Egami, 1980; Hoornbeek and Burke, 1981; Streisinger *et al.*, 1981; Chourrout, 1982b; Taniguchi *et al.*, 1986; Kavumpurath and Pandian, 1992). All the listed treatments have both advantages and disadvantages.

(a) X-rays and gamma (χ) rays

X-rays and χ-rays inactivate sperm by inducing breaks in the chromosomes and have good penetration, therefore they permit the treatment of large volumes of sperms. Stanley, (1983), reported that X-ray treatment reduces the activity of sperm. However, Thompson *et al.*, (1981), found that plaice sperm irradiated with ^{60}Co χ-rays and maintained at 0°C retain activity for up to 5 days. χ-ray treated sperm are less effective than normal sperm at high dilution (Chourrout *et al.*, 1980). Even after high doses of χ- or X-rays irradiation, gynogenetic embryos sometimes show residual paternal characteristics or chromosome fragments (Ijiri, 1980; Chourrout *et al.*, 1982; Onozato, 1982).

(b) Ultraviolet (UV) light

Ultraviolet (UV) light is a relatively hazard-free treatment, which is very easy to work with. It damages chromosomes mainly by inducing thymine dimers which result in damaged chromatin. Photo-reactivation, which occurs in the presence of visible light, can be cause for caution as it can repair this damage, especially in small transparent, fish eggs (Ijiri and Egami, 1980). UV light sources are easy to set up and are relatively inexpensive (Nace *et al.*, 1970; Stanley, 1976; Streisinger *et al.*, 1981; Chourrout, 1982b; Onozato and Yamaha, 1983; Taniguchi *et al.*, 1986; Kavumpurath and Pandian, 1992; Arai *et al.*, 1992). UV light can also be used safely but this is a mixed blessing as due to its low capacity to penetrate water only small volumes of sperm in thin layers can be treated at a time.

CHEMICALS

DNA disrupting chemicals like toluidine blue (Briggs, 1952; Uwa, 1965), ethyleneurea (Jones *et al.*, 1975), and dimethylsulfate (Tsoi, 1969;) have all been successfully used to induce gynogenesis in fish and amphibians.

1.5.5 Androgenesis

All paternal inheritance in fish has not been studied as widely as gynogenesis. Spontaneous androgenesis has occasionally been observed in interspecific crosses and androgenetic grass carp *Ctenopharyngodon idella* result, albeit at low frequencies, from crosses of female carp *Cyprinus carpio* with male grass carp (Stanley and Jones, 1976).

1.5.5.1 Oocyte inactivation

Possible irreversible damage sustained by essential maternal constituents such as messenger RNA (mRNA), mitochondrial DNA and cytoplasmic organelles in the oocyte are factors which constrain the use of radiation treatment to as a means to inactivate maternal chromosomes. Purdom, (1969), and Arai *et al.*, (1992), working with loach *Misgurnus fossilis* and masu salmon *Oncorhynchus masou* have successfully produced androgenetic haploids using ionising radiations, although they reported that androgenetic haploids have an increased incidence of severe problems compared to gynogenetic haploids, possibly due to damage to cytoplasmic components, caused by the radiation treatment. However, Purdom, (1969), working with the plaice *Pleuronectes platessa* was unable to show any difference in the viabilities of gynogenetic and androgenetic haploids. Scheerer *et al.*, (1986), using ^{60}Co χ-radiations, produced diploid androgenetic rainbow trout, using sperm from both inbred and outbred sources. Survival of androgenetic diploids from inbred and outbred sperm sources was similar and significantly below that of outbred controls.

UV light provides an alternative means of inactivating the egg genome and is attractive in that its low penetration could be used to minimise damage to egg cytoplasmic components. The first use of UV light is recorded in studies with amphibians (Gillespie and Armstrong, 1980; 1981), where the transparency of the egg, and the characteristic of the egg pronucleus of amphibian eggs to orient

toward the animal pole after fertilisation was used to advantage. In some fish species the opacity of the eggs and the lack of any particular orientation (Devillers, 1961) negates the use of UV light but it may be useful in species with small transparent eggs, or those whose pronuclei show a specific orientation. UV irradiation has been shown to be successful in genetically inactivating eggs of the loach *Misgurnus anguillicaudatus* (Arai *et al.*, 1992).

1.5.5.2 Physical treatments

Physical treatments of eggs has also been used as a means of inducing androgenesis and is attractive in that it is less deleterious to the cytoplasmic components than radiation treatments. For example Gervai *et al.*, (1980), found a high frequency of androgenetic haploid carp embryos after a cold-shock treatment initiated shortly after fertilisation. Androgenetic haploid amphibians are occasionally produced when recently fertilised eggs are subjected to cold (Frankhauser, 1945) or pressure shock (Elinson and Briedis, 1981; Briedis and Elinson, 1982).

Overripe rainbow trout eggs show a tendency to androgenetic haploid development (Yamazaki, 1983).

1.5.6 Identification of Gynogenetic and Androgenetic Diploids

It is imperative to exclude genetic participation of the egg (in androgenetic diploids) or sperm (in gynogenetic diploids) in experiments of this kind. This evidence could be obtained by one of several methods.

1.5.6.1 Hybrid Markers

In gynogenesis studies a simple option is to use sperm from a related species to activate the egg and initiate development (Nace *et al.*, 1970; Allen and Stanley, 1979). Unsuccessful gynogenesis would in this case result in a non viable or

morphologically or biochemically recognisable hybrids (Stanley, 1976a). The availability of suitable sperm-donor species is imperative as it eliminates having to rear genetically distinct strains of donors, or of biochemical analysis of the test species to identify suitable, biochemically identifiable, donors. Incompatibility between the sperm and the egg components may limit the use of eggs of a related species for androgenesis studies. However, Stanley and Jones, (1976), found that androgenetic grass carp, derived from carp eggs fertilized by grass carp sperm, were morphologically normal.

1.5.6.2 Allelic Markers

Within a species, colour, phenotypic, or biochemical markers can be utilised as a suitable determinant of gynogenesis or androgenesis. Irradiated sperm carrying dominant colour alleles were used to fertilise eggs that contained the recessive alleles, and absence of the dominant colour was used as proof of all-maternal inheritance in rainbow trout (Chourrout *et al.*, 1980) and zebrafish (Streisinger *et al.*, 1981). Nagy *et al.*, (1978) and Gromelsky *et al.*, (1992), fertilized eggs of homozygous recessive carp which had a scattered (ss) scale pattern phenotype, with irradiated sperm from normal scale patterned males; gynogenesis was proved by all the progeny exhibiting the scattered (ss) scale pattern. The former group also used a biochemical marker, variation at the transferrin locus, to provide proof of gynogenesis.

1.5.6.3 Genetic Markers

Genetic markers can help determine whether suppression of the first mitotic division or prevention of polar body extrusion was responsible for diploid gynogenesis (Gomelsky *et al.*, 1992). Prevention of the first mitosis would result in the progeny of a heterozygous female being all homozygous. Suppression of first polar body extrusion, although unlikely as it occurs prior to ovulation (Cherfas, 1981; Purdom, 1970), would result in almost entirely heterozygous progeny for genes near the centromere, decreasing to 66% heterozygotes for

genes segregating randomly in relation to their centromere. Prevention of second polar body expulsion would result in predominantly homozygous progeny for genes near the centromere, with the proportion of heterozygotes increasing as the distance from the centromere increases. For genes segregating randomly in relation to their centromere 66% of the progeny should be heterozygous (Nace *et al.*, 1970).

1.5.7 Sex Control of homozygous diploid fish

One of the main reasons for commercial interest in gynogenesis and androgenesis is the potential for the production of monosex populations. In female homogametic species (XX), all the gynogenetic progeny should be female. In species with female heterogamety (XY), both males and females may be produced. The sex of gynogenetic progeny can therefore provide information about the sex-chromosome system prevalent in that species. All female progeny as a result of a gynogenesis in grass carp, is consistent with a female homogametic system (Stanley, 1976b). Similar results in carp (Nagy *et al.*, 1978, 1981; Nagy and Csanyi, 1984;), coho salmon, *Oncorhynchus kisutch* (Refstie and Donaldson, 1982), pink salmon *O. gorbuscha* (Maximovich and Petrova, 1980), and rainbow trout (Chourrout, 1982) also indicate female homogamety in these species. When male progeny are also observed after gynogenesis (Purdom and Lincoln, 1973), it is interpreted as suggesting female heterogamety in that species. Streisinger *et al.*, (1981) found considerable variation in the sex ratio of various clones obtained when producing homozygous diploid clones of zebrafish by gynogenesis. Interestingly some clones derived from single homozygous females were predominantly male which is inconsistent with either a female- homogametic or heterogametic system, but could derive from an autosomal sex-determining system or environmentally influenced sex determination.

In carp and most salmonids, which are a male heterogametic species, androgenesis, and subsequent diploidization by inhibition of the first mitotic division, should yield in an equal proportion (50%) of XX and (50%) YY progeny. The XX individuals would be homozygous females and the YY individuals would be homozygous males, the latter producing an all-male generation when crossed with normal females. Hormonally induced, sex-reversal studies, with gold fish (Yamamoto, 1975), medaka (Yamamoto, 1964), and coho salmon (Hunter *et al.*, 1982), have demonstrated that YY males are viable. In male homogametic species androgenesis should result in production of all-male progeny. Gillespie and Armstrong, (1981), working with axolotls, a male homogametic salamander, produced androgenetic diploid males by inhibiting first mitosis.

Androgenesis and gynogenesis in fish can be used as a direct route to producing monosex populations for aquaculture purposes, however the resulting progeny could be expected to exhibit inbreeding depression leading to poor viability, survival and growth rates when compared with normal fish. However the use of YY males or sex-reversed XX males in male heterogametic species, can be used to produce all-male (monosex) populations that compare favourably with the inbred strains mentioned above. The use of gynogenesis to produce monosex populations for release in the wild is problematical, as the fish are reproductively viable and being genetically uniform and can establish an undesirable inbred "***monoculture***" in nature (Streisinger *et al.*, 1981).

1.5.8 Production of Inbred Lines of Fish

The ability to manipulate fish breeding to rapidly produce both inbred lines of fish and monosex populations, is of special interest to both basic biological research (Streisinger *et al.*, 1981; Schultz and Schultz, 1982), and aquaculture. F1 crosses between inbred lines have the potential to provide uniform test animals

in growth and disease studies (Thorgaard, 1986) and as has been demonstrated in plants, yield improved strains by capitalising on the hybrid vigour occurring when two desirable homozygous lines are crossed (Stanley and Sneed, 1974; Purdom, 1976). From a commercial view point too, inbred lines can be used as a practical means of preserving desirable genetic characteristics whilst eliminating less desirable factors or unwanted variability (Purdom, 1970). Gynogenesis can be achieved by either second polar body retention, which leads to a degree of homozygosity among offspring depending on the amount of crossing over occurring at meiosis, or by suppression of the first mitotic division, which results in greater inbreeding depression and therefore is more difficult to achieve. Individuals of an inbred line produced by polar body retention will be similar (isogenic) as they will remain heterozygous for the same loci (Nagy and Csanyi, 1982). However when a homozygous diploid gynogenetic zebrafish, produced by inhibition of first mitosis, was subjected to polar body retention, all the offspring were identical homozygous diploids (Streisinger *et al.*, 1981).

A novel application of gynogenesis is as a means of effecting gene transfer, in cases where sperm inactivation is incomplete and some residual paternal inheritance occurs (Thorgaard *et al.*, 1985), however the use of this is limited.

Androgenesis, followed by suppression of the first mitotic division, would be another route to the production of homozygous diploids. Androgenesis could permit a rapid turnover of generations as opposed to gynogenesis as in some species males reach maturity more rapidly than females, also the ability to cryopreserve sperm allows an inbred line to be maintained frozen, and recovered through androgenesis (Stoss, 1983). Another application of androgenesis could be in investigating the effects of mitochondrial genotype on performance, as mitochondrial DNA in animals is assumed to be maternally

inherited (Avise and Lansman, 1983) although this assumption is challenged (Gyllensten, 1991).

1.5.8.1 Inbreeding Depression

The increased probability of harmful recessive alleles becoming homozygous and the associated reduction in reproductive and somatic efficiency of inbred individuals is termed inbreeding depression. Androgenesis and gynogenesis are very powerful methods for increasing the rate of inbreeding. In fish crossing-over occurs once in every ten chromosomes during meiosis, therefore there is an estimated inbreeding in gynogens (siblings) of 95%. This is equivalent to 14 generations of sibling-sibling (brother-sister) mating (Purdom, 1970). Most commercial species mature after 2 or 3 years, so normally it would take 30-40 years of back crossing to achieve the equivalent level of inbreeding produced by one run of gynogenesis or androgenesis. So, the fact that gynogenetic fish show evidence of inbreeding depression (Cherfas, 1981), is not surprising.

Reduced survival of gynogenetic fish has been reported for plaice (Purdom, 1969), carp (Nagy *et al.*, 1978), zebrafish (Streisinger *et al.*, 1981), coho salmon (Refstie and Donaldson, 1982) and rainbow trout (Foisil and Chourrout, 1992). Growth rate should be less strongly influenced by inbreeding, than survival and reproductive efficiency (Lerner, 1954), and this has been supported by reports on grass carp (Stanley and Sneed, 1974), where gynogenetic individuals have shown satisfactory growth rates. In contrast Refstie and Donaldson, (1982) reported a reduction in growth rate of gynogenetic coho salmon. An increase in developmental abnormalities has been reported in gynogenetic individuals. Percentages of abnormal spinal curvature of gynogens was increased in brown trout (Purdom, 1969) and rainbow trout (Chourrout, 1980), and frequency of abnormal gonadal development has been observed in gynogenetic carp (Gomelsky *et al.*, 1979). Treatment effects, as have been

observed for triploids, could also be responsible for some of the developmental abnormalities and reduced survival of gynogens. Progeny of gynogens showed better survival and viability than their parents in both carp (Cherfas and Ilyasova, 1980) and zebrafish (Streisinger *et al.*, 1981).

The effects of the treatment (heat shock, cold shock, pressure shock etc) need to be ascertained prior to gynogenesis being used as a method for determining the degree of inbreeding depression for different characters in fish and the *genetic load* (the number of harmful recessive alleles normal individuals carry) of a species.

1.5.9 Sex Inversion

Another approach to produce monosex populations of fish is to induce sex inversion using the corresponding hormones, androgens for males and estrogens for females, and mating the resulting sex-reversed fish with the corresponding sex, resulting in all-male or all-female populations. 17-α Methyltestosterone was fed to rainbow trout fry (Bye and Lincoln, 1986), and zebrafish embryos (Streisinger *et al.*, 1981) to produce phenotypically male broodstock of female genotype which were used to fertilise eggs from normal or gynogenetic diploid females resulting in all-female progeny.

1.6 LARVAL REARING

Maintaining, inducing sexual maturity and spawning of adults, and the subsequent rearing of offspring to maturity are three areas that together constitute closing the life cycle of any species and for fish also all three areas are of equal importance especially for biotechnical research and commercialisation. The area of larval rearing is discussed in this literature review in general and specific factors of relevance to laboratory based larval rearing are reviewed.

Rearing of offspring in fish as opposed to most mammals, commonly requires relatively complex and intensive technology that varies from species to species. The importance of different factors varies considerably, with different species and survival rates required, therefore few generalisations can be made between species. The important factors include feeds, and physical factors such as water quality, temperature, and photoperiod.

1.6.1 Feeds

First feeding (weaning) is a critical time for fish larvae and the feed is the single most important factor in larval rearing. They can be broadly classed as dry food, live food and frozen live food.

1.6.1.1 Dry feeds

Dry foods such as artificial powdered (commercially available) diets and microencapsulated diets, are convenient to store and administer to the fish, and are the ultimate goal for larval rearing, however except for a few species including snapper (Bell *et al.*, 1991), speckled dace *Rhinichthys osculus* (Kaya, 1991), carp (Alami-Durante *et al.*, 1991), and paddlefish *Polyodon spathula* (Kurten *et al.*, 1992), they have been shown to be sub-optimal, resulting in very low (Awaiss *et al.*, 1992; Roesch and Segner, 1990; Rottmann *et al.*, 1991; Fermin and Bolivar, 1991) and often no larval survival (Walford *et al.*, 1991; Kestemont and Stalmans, 1992).

Zebrafish larvae are visual feeders (Laale, 1977) and attempts to raise them on a minimum of live food (Paramecia Day 1-3, followed by powdered food) resulted in a mean survival to Day 14 of 38% and this decreased survival was also observed for older fry with a survival rate of only 29% recorded for fry from the fourth to the twentieth week of age (Von Hertell *et al.*, 1990), highlighting the importance of live food for this species.

1.6.1.2 Live feeds

Live foods include zooplankton (brine shrimp *Artemia salina, Moina macrocopa, Daphnia spp.* and rotifers *Brachyionus spp.*), ciliates (*Paramecia, Tetrahymena* and *Euplotes*), and nematodes (micro-worms *Panagrellus spp., Tubifex tubifex*), with artemia being the most commonly used live food (Huang and Hu, 1989; Eda *et al.*, 1990a; 1990b; Fermin and Bolivar, 1991; Rottmann *et al.*, 1991; Fermin, 1991) followed by Rotifers (Huang and Hu, 1989; Walford *et al.*, 1991; Awaiss *et al.*, 1992; Battaglene and Talbot, 1992), probably due to their ease of culture and ability to be enriched (Dhert *et al.*, 1992; Tamaru *et al.*, 1993). Eaton and Farley, (1974b) have demonstrated extremely efficient, though laborious, rearing of zebrafish larvae using *paramecia* and *artemia* as live food, with survival rates of >95% reported. Less labour intensive regimes of rearing zebrafish on live food consisting of infusoria (a crude culture including a mixture of protozoans and ciliates) result in a lowering of survival, with 15-20% being achieved (H. W. Khoo per comm).

Live foods are desirable due to the good larval survival and growth achieved with their use but they present the problems of culturing, maintaining and harvesting the organisms fresh for each feeding. In actual fact, they add an additional step in the larval rearing protocol and are least desirable for these reasons.

1.6.1.3 Frozen feeds

Frozen live foods are a useful compromise which can work quite well for some species, especially as a substitute for live food (Villegas and Lumasag, 1991; Kestmont and Stalmans, 1992) as they can be stored frozen for long periods and most are commercially available.

Other factors relating to feed that can influence the outcome of reared larvae are, the size of the particle that the larvae can ingest can be of importance to

ultimate survival and so to can the feeding characteristics of the species such as cannibalism can be of enormous significance (Fermin and Bolivar, 1991; Parazo *et al.*, 1991). The feeding regimes are of importance especially when stocking densities are high (Holm *et al.*, 1990).

1.6.2 Physical Factors

This encompasses a range of factors including water quality, temperature, and light.

1.6.2.1 Water Quality

Water quality is obviously tremendously important in any aquaculture or fish rearing set up and zebrafish are no different (Kahn, 1994). This is linked to the type of system being utilised for the rearing. Bio-filtration has been successfully used to maintain water quality suitable to sustain very high densities of major Indian carp fry (Prakash *et al.*, 1990) and for large scale rearing of zebrafish larvae (Mullins *et al.*, 1994). Alkaline phosphatase has been demonstrated to be closely related to the bacterial count, and therefore is considered a indicator of water quality (Sidik *et al.*, 1990).

Optimisation of water quality and feeding resulted in a 2- to 4-fold increase in stocking densities of Japanese flounder (Honda *et al.*, 1991). Ng *et al.*, (1992), found a system of ponds, aquatic plant tanks and gravel filters to be adequate for recycling effluent from an ornamental fish farm, in terms of physical, chemical and microbiological parameters. The effectiveness of UV irradiation of water in controlling fish pathogens by decreasing bacterial density makes it a desirable feature for any culture facility (Sugita *et al.*, 1992).

1.6.2.2 Temperature

Temperature is an important factor affecting larval growth and yolk utilization, as the time to starvation in unfed larvae of a number of species occurs earlier at higher temperatures (Fukuhara, 1990). The survival times and first feeding capabilities of *O. niloticus* hatchlings are also temperature-dependent (Rana, 1990b). Temperature can induce meristic variation in medaka (Ali and Lindsey, 1974), and in goldfish, cool incubation conditions can increase the incidence of developmental abnormalities (Wiegand *et al.*, 1989). These results suggest higher temperatures being preferable, however, the optimal temperature need not be the highest tolerable as has been demonstrated for Atlanic salmon *Salmo salar* (Jon-Siemien and Carline, 1991), and some species do not exhibit any correlation between developmental rate and temperature (Beacham and Varnavskaya, 1991). Other factors such salinity can alter the effects of temperature (Watanabe *et al.*, 1993).

1.6.2.3 Light

Extended photoperiod is generally preferable for visual-feeding juvenile fish however swim bladder inflation in Australian bass *Macquaria novemaculeata* larvae was significantly affected by the photoperiod with the normal rate of inflation of 70% being totally inhibited by continuos exposure to fluorescent light (Battaglene and Talbot, 1990). Silver bream *Sparus sarba* larvae showed significant differences in their ability to capture prey at different light intensities (Huang and Hu, 1989).

1.6.3 Conclusion

Being visual feeders survival of danio larvae raised on dry powdered foods supplemented with infusoria is very low with survival rates to maturity of 20% usually achieved (H.W.Khoo per. comm.). Therefore, the two most important aspects of a successful rearing operation for danio larvae is the supply of

"***squeaky clean***" water (C. Nusslein-Volhard quoted in Kahn, 1994), and an abundant supply of live food of the appropriate size in relation to the age of the larvae. *Paramecia spp* and brine shrimp supplemented with powdered food have been identified as suitable starter and secondary food respectively for danio larvae and fry (Eaton and Farley, 1974b; Mullins *et al.*, 1994). Production of relative pure cultures of paramecia however is a laborious and demanding task (Eaton and Farley, 1974b), and though alternate culture techniques for paramecia have been described (Mitchell, 1991; Westerfield, 1993), they are relatively labour intensive.

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1 MEDIA

2.1.1 Hank's Balanced Salt Solution (HBSS)

HBSS used for preparation of danio and medaka sperm suspensions and for collection of medaka ovulated oocytes was prepared from powdered HBSS (Sigma, MI, USA) dissolved in freshly purified water, and supplemented with 2 mM sodium bicarbonate. The pH was adjusted to 7.4 before filter sterilisation (0.22 μm; Millipore, MA, USA).

2.1.2 Fish Ringer's Solution

Fish Ringer's solution used for culturing chorion damaged danio embryos was prepared according to Ginsberg (1963), as follows: 6.50 g NaCl, 0.246 g KCl, 0.20 $NaHCO_3$, 0.30 g $CaCl_2$ brought up to a volume of 1 litre with reverse osmosis (RO) water.

2.1.3 Earle's 199 Medium

E199 media used for *in vitro* maturation (IVM) of medaka oocytes was prepared from powdered Earle's 199 Medium (Sigma) dissolved in purified water. Additional supplements included 2% bovine serum albumin (BSA), and 2 mM $NaHCO_3$. The pH of the prepared media was adjusted to 7.2 before filter sterilisation (0.22 μm; Millipore).

2.1.4 Benzocaine (Ethyl-4-aminobenzoate)

A benzocaine stock solution of 100 g/L in Milli-Q water was prepared by initially dissolving 1g of benzocaine (Sigma) in 100 ml of ethanol (as it is

insoluble in water), and the stock solution was stored in a dark bottle for up to a year. The stock solution was diluted in tank water immediately prior to use, and used at a final concentration of 25 mg/L.

2.2 MAINTAINING FISH STOCKS

2.2.1 *Danio rerio* and *D. frankei*

The zebrafish *Danio rerio* is a tropical representative of the family Cyprinidae, which is readily available, inexpensive, hardy, small sized and has a life cycle of approximately 3 months. It is an oviparous species which produces large (1 mm), non-adherent, transparent eggs which hatch out in approximately 72h at 26°C. The females of this species display high fecundity and produce hundreds of eggs on a weekly basis, with spawning usually occurring with the first 90 min of the photoperiod. Zebrafish display a phenotype of metallic blue stripes that run along the lateral line of the body (see Plate 1.1).

The leopard danio *D. frankei* is a related species to *D. rerio* with similar characteristics (see review by Laale, 1977), however the striped phenotype of the latter is replaced by rows of spots (Plate 1.1), hence the common name (leopard danio). A color mutant of *D. frankei* called golden danio was identified and used for some chimeric experiments.

Zebrafish, leopard and golden danios were maintained using identical methods.

2.2.1.1 Tanks

Two systems of tanks were employed for this study.

Static System

Individual 60 L glass tanks filled with dechlorinated tap water with under-gravel filters overlaid with gravel. The temperature of the water was controlled with a thermostatically controlled submersible aquarium heater (200 Watt; Rena, France) in each and stocked with not more than 50 adult fish per tank.

Recirculating Flow-Through System

A 300 L Recirculating Flow-Through System consisting of a common reservoir connected with six 20 L acrylic tanks was designed and purpose built. Each tank had a continuos flow of 50-80 L per hour (which could be controlled) of water from the reservoir. Fish could be stocked at densities of greater than 100 per 20 L tank. The water in the reservoir was aerated with air stones and the temperature was maintained at 26°C with two 300 Watt submersible, thermostatically controlled aquarium heaters (Rena). Water from the reservoir was filtered through a Biological Canister filter (Fluval 403; Italy) and rated at 1200 L per hour, and fed via plastic taps to each tank , an outlet was connected to the other end of each tank and the overflow was collected in a return channel that drained back into the reservoir. The outlet from each tank was covered with a plastic mesh of an appropriate size to prevent the fish escaping into the reservoir. A UV water sterilisation unit (RUV 30 Watt; Rena) was connected independently to the reservoir and run for 5 hours (h) each day. To comply with Biohazard regulations and to rescue fish in the event of accidental breakage or overflow, tanks were placed in plastic 40 L trays. Each tray held two tanks and had a mesh covered outlet that also drained into the return channel. Apart from removal of the algae from the walls of the tanks, all the maintenance (water changes *etc*) were performed on the reservoir.

2.2.1.2 Photoperiod

Fish were exposed to a 14:10 hour, Light: Dark cycle with the lights being switched on at 0900h. The tanks were covered with an appropriate light proof cover to prevent disruption of the photoperiod by external light.

2.2.1.3 Water Quality

It is vital for breeding that the water quality is maintained by frequent changes, and regular monitoring of pH, ammonia, nitrite, nitrate, hardness *etc* was carried out. The water changes for the glass tanks was performed fortnightly, using a commercially available gravel cleaning siphon. No more than 20% of the volume of water was replaced with fresh dechlorinated tap water at each change. The algae that sometimes grew on the tank walls was removed with a kitchen scourer reserved for that express purpose. The water changes for the flow through system were performed on the reservoir on a monthly basis. Any debris visible on the floor of the reservoir was siphoned off, along with 10% of the total volume of the system, which was replaced with dechlorinated tap water. Evaporation was significant in the flow through system and this loss was topped up with reverse osmosis (RO) water to prevent the build up of salts which would result if tap water was used for the top up. The pH of the water was monitored with a hand held pH meter (Hanna Inst.; Japan) and was maintained in the range between pH 6.8 and 7.5. Aeration in the glass tanks was provided by the under-gravel filter, and for the flow through system the reservoir and each tank were aerated with individual air stones fitted to an aquarium air pump, to prevent a layer of scum forming at the surface.

2.1.2.4 Food

Two nutritional regimes were followed, based on the dietary requirement of the fish.

MAINTENANCE DIET

Basic feeding for maintaining fish stocks was either tropical fish flakes (Wardley, NJ, USA) or floating fish pellets (code B-2; Nisshin Flour Milling Company, Tokyo, Japan) fed *ad libitum* twice a day via an automatic feeder or by hand.

BROODSTOCK DIET

Brood stock were fed twice a day as above and were fed additionally with live or snap frozen brine shrimp *Artemia salina* in the afternoon for a week prior to setting up spawning.

2.2.2 *Oryzias latipes*

The Japanese rice fish *Oryzias latipes* also known by the common name medaka (Plate 1.1) is a small oviparous teleost which possesses a number of attributes desirable in a piscine model species, such as large (>1 mm) transparent eggs, a short life cycle of 3 months. It also displays the rather unique characteristic (for a fish species) of a precise 24h ovulation cycle, once spawning commences, which increases the appeal for biotechnologists especially as *in vitro* maturation has been standardised for this species (for a review on medaka see Ozato *et al.*, 1989). Medaka displayed different requirements to zebrafish in regards to both water temperature and food.

2.2.2.1 Tanks

Medaka were stocked in 60 L glass tanks with under gravel filters, with the photoperiod and water quality requirements similar to those for danios. However, medaka did not tolerate extended duration's of tropical temperatures (26-28°C) therefore the temperature was maintained at 22-23°C for stocking, while for breeding fish it was raised to 26°C.

2.2.2.2 Food

Basic food requirements were provided with the same fish pellets used for the danios fed twice daily, with an additional feed of snap frozen Daphnia *D. pulex* when the fish were spawning.

2.3 BREEDING FISH

The requirements of fish alter dramatically when they need to be conditioned to spawn and the species in this study were no exception.

2.3.1 Danios

Danios (both zebra and leopard) generally spawn within the 90 min following the onset of light, and the embryos hatch out in ~72h at 26°C. Brood stock for both natural mating and artificial insemination (AI) were set up by stocking the fish in a 1:1 or 3:2 male:female ratio and introducing a spawning substrate such as a spawning mop made out of strands of green wool.

2.3.1.1 Natural Spawning

For natural spawning in the glass tanks, a clear acrylic collection container with a black plastic mesh (garden trellis), of a suitable size to prevent the adults from passing through, fitted a few millimetres above its base was used to prevent cannibalism of the spawned eggs. The spawning mop was placed in the collection container. The fish usually spawned in the spawning mop and the fertilised eggs sank to the bottom of the collection container from where they were recovered. For group matings in the flow through tanks the brood stock were placed with the spawning mop inside plastic 3 L cereal containers (nuptial chamber) which had the base replaced with the black mesh mentioned previously. The spawned eggs fell through the mesh attached to the base of the nuptial chamber to the bottom of the tank from where they were collected by siphon. When fish were required to be mated in pairs, the same procedure was

followed with the addition of a tight fitting collection chamber being coupled to the bottom of the nuptial chamber to isolate the spawned embryos.

2.3.1.2 Artificial Insemination (AI)

For AI the fish were observed from the onset of the light phase and spawning females were netted and isolated in beakers of tank water. Sterile 230 mm glass pasteur pipettes, with the tapering end cut to give an approximate inner diameter of 1.5 mm, were used for collection of oocytes; uncut pipettes were suitable for collection of the milt. The tips of both cut and uncut pipettes were heat polished over a flame to prevent any jagged edges which might have injured the fish.

A preset volume (usually 50-100 µl) of Hank's Balanced Salt Solution (HBSS) pH 7.4 (for preparation of media see section 2.1.1) was pipetted into a disposable 36 mm plastic cell-culture dish (Disposable Products, SA, Australia). Males were anaesthetised with Benzocaine (for preparation see section 2.1.4) at a dose of 25 mg/L, and the region around the genital pore was dried carefully with a paper towel, and gentle pressure along the lateral line was applied with the thumb and forefinger of the left hand. The milt (semen) was collected in a pasteur pipette and expelled into the drop of HBSS in the culture dish . A volume of ~2-4 µl milt was routinely collected from each male and a final sperm concentration of approximately 10^8 sperm/ml was attained by stripping an appropriate number of males (typically 3-5).

Females were anaesthetised, dried and stripped in the same way as males and the ovulated oocytes were collected in the egg collection pipette. The ovulated oocytes were released into the sperm suspension and the gametes were mixed. Activation of eggs and sperm suspension was achieved by dilution with tank water after which fertilised eggs were placed on a warming tray set at 26°C to

commence development. After 5 min the dish containing fertilised eggs was filled with tank water to prevent desiccation of the embryos.

2.3.2 Medaka

2.3.2.1 Natural Spawning

Medaka usually spawn in the hour preceding the onset of the light phase, and once they begin to spawn they usually follow a 24h ovulation cycle for 10 to 14 days which helped with identification of spawning females. The fish were stocked at male:female ratios of 1:1. No spawning substrate was required as medaka females are internally inseminated *via* the intromittant organ of the male, and subsequent to fertilisation the embryos, which clump together due to the filaments on the chorion (egg shell), were released from within the body cavity and could be observed in a cluster like a "*bunch of grapes*" just below the ovipore (see Plate 1.1), from where they were collected. The embryos hatch in approximately 10 days at 26°C.

2.3.2.2 Artificial Insemination

Spawning females, with eggs attached near the ovipore, were identified the day previous to when they were required and isolated in 250 ml glass beakers that were maintained at 26°C. At the appropriate time (~1h) before the onset of the light phase the males were sacrificed and the testes dissected out and placed in 100 µl of HBSS (pH 7.4) in a culture dish, in which they were teased with watchmaker forceps and allowed to stand for 5-10 min to allow the sperm to disperse out of the testes. The sperm suspension was collected with care being taken to avoid collecting the testicular debris and placed on ice. The spawning females were then sacrificed and ovulated oocytes were transferred, with a sterile pasteur pipette similar to the oocyte collection pipettes used for Danio oocytes, to the sperm suspension. Fertilisation was effected by diluting the

sperm suspension 1:1 with Milli Q water. After 5 min the dish was filled with filtered tank water and incubated at 26°C.

2.3.2.3 In Vitro *Maturation*

For microinjection into the germinal vesicle (GV) of pre-ovulatory medaka oocytes, procedures described by Ozato *et al.*, (1986) were followed with minor modification, briefly; oocytes were collected from spawning females sacrificed 9h prior to fertilisation and placed in Earle's 199 medium supplemented with 2% bovine serum albumin (BSA) and 2 mM $NaHCO_3$ (pH 7.2). Oocytes of approximately 1000 μm diameter, with GV's near the surface of the peri-vitelline membrane were used for microinjection. After micromanipulation the oocytes were cultured in 4-well tissue culture plates on the warming tray. At the designated fertilisation time the oocytes that had failed to ovulate spontaneously had their follicle manually removed with fine forceps. The oocytes were subsequently inseminated with a sperm suspension, fertilized, and cultured at 26°C.

2.4 REARING OF DANIO LARVAE

2.4.1 Production of Live Food

Suitable practical and relatively less labour intensive methods adapted or developed for either small or large scale production of relatively pure cultures of live food are described along with modifications.

2.4.1.1 *Brine shrimp* Artemia salina

Brine shrimp nauplii were produced by procedures described (Axelrod *et al.*, 1987) with minor modifications, briefly; *Artemia* eggs were purchased from a commercial source (San Francisco Bay Brand, California, USA). For small scale production of brine shrimp nauplii adequate for the maintenance of 100-200 larvae, 500 ml beakers were filled with Milli-Q water and the required level of

salinity was achieved by the addition of 15 g of table salt. Brine shrimp eggs (0.5-1 g) were added to this solution which was vigorously aerated with air stones and maintained at 24-26°C. The nauplii usually began to hatch within 20h and could be observed swimming in the water column with the naked eye.

To facilitate harvesting of pure populations of nauplii without the unwanted collection of dead eggs and shells, the aeration was turned off allowing the immotile fraction to either settle and the bottom of the container or to float to the surface, facilitating the collection of hatchlings from the water column. The brine solution was removed by straining the nauplii through a 50 μm nylon mesh and returned to the beaker. The collected brine shrimp were washed 3x and resuspended in tank water and fed to the larvae. Each culture lasted for approximately 3 days after which the equipment was washed thoroughly, dried and used to initiate a fresh culture. As the eggs hatch out within 24h the cultures could be set up as and when required.

2.4.1.2 Paramecia P. caudatum

Lettuce bought from a local greengrocer was washed and one or two leaves were grated and blanched by microwave treatment (30 seconds) in approximately 100 ml tap water. After cooling to RT the blanched lettuce was transferred to a rectangular disposable plastic container (the type used to package take away food) which was filled with approximately 400 ml (final volume) of either tap water or Milli-Q water. 10-15 ml of a paramecium starter culture (Southern Biological Supply, Vic, Australia) was added to the container and the containers were kept illuminated at RT, for 4 to 6 days, by which time they attain concentrations suitable for harvesting.

Cultures with paramecia at densities of 1000 /ml were harvested by filtering through a 100 μm mesh to trap the particulate vegetable matter (the paramecia pass through the mesh) and are fed directly to the larvae. The vegetable matter

was returned to the culture container which was topped up with enough Milli-Q water to replace the volume removed. Depending on usage the cultures were passaged every 7-14 days in a similar manner with an aliquot from an established culture used to seed fresh cultures.

2.4.1.3 Microworms (Anguilla silusiae)

Microworms purchased from a local pet shop were cultured in infant cereal formula (such as Farex). Formula was hydrated with Milli-Q water and mixed till it attained a pasty consistency and transferred to containers which had a lip or shoulder in the walls (containers for some brands of margarine) to a depth of no more than 10 mm. One ml of starter culture was spread over the surface of the formula paste, the cover of the container was pierced to allow ventilation and the containers were maintained at RT. Within 3-4 days the multiplying microworms could be observed at the surface of the paste and migrating up the sides of the container. The purest population of worms, relatively free of the formula which pollutes the water, was usually found in the lip on the walls of the container and was collected using a spatula. The worms collected were fed directly to the larvae or fish. Fresh cultures were initiated fortnightly seeded with an aliquot harvested from an established culture.

2.4.2 Feeding protocol

2.4.2.1 First (Starter) Food

Danio larvae hatch in ~72h (Day 3 at 26°C) after fertilisation and the yolk resources are exhausted by Day 5, therefore first feeding was commenced on Day 4. Larvae were maintained in containers of varying sizes depending on the density and age of the larvae. Smaller containers were utilised to optimise the concentration of paramecia for young larvae and they were replaced with larger containers as the larvae grew. Small batches of 15 or fewer larvae were initially reared in 250 ml plastic containers (Disposable Products) in volumes of 200 ml

(from Days 4-15), and larger batches of 40-50 larvae were reared in 600 ml containers. Approximately 10 ml of the harvested culture of paramecia was added per 250 ml container, (larvae in containers with larger volumes were fed accordingly), at least twice daily.

2.4.2.2 Secondary Live Food

The larvae were raised entirely on paramecia until Day 11-12, and from then on the larvae were weaned onto *Artemia* nauplii. Any uneaten brine shrimp were siphoned off and the volume lost was replaced with tank water. By Day 15 when most of the larvae were feeding entirely on brine shrimp fed twice daily (the belly of larvae that have eaten brine shrimp appear orange), paramecia were stopped. Microworms *Anguilla silusiae* were occasionally used as a substitute for brine shrimp nauplii however they were not preferred as even small traces of the formula that was transferred to the larval containers polluted the water.

2.4.2.3 Supplemental Dry Food

Dry powdered fish food (code A1, Nisshin Flour Milling Company, Japan) was provided as a supplemental food from Day 18, by which time the larvae were transferred to either 2L or 4L containers. Water was replaced daily and any dead fish were removed promptly. By Day 30 the fry had usually developed their adult markings and were transferred to glass tanks. Typically 20-30 and no more than 50 fry were stocked per 60 L tank, in which they were maintained similarly to adults and raised to maturity.

2.5 ANALYTICAL METHODS

2.5.1 Production of transgene constructs

The human metallothionein IIA promotor/porcine growth hormone fusion gene (pHMPG.4; Vize *et al.*, 1988) and the RSV-*lacZ* transgene (R. Keough per

comm) were provided by the Dept. of Biochemistry, University of Adelaide. Brief details of the preparation of the most used transgene (pGL2) that was carried out by the author are provided.

The entire 6.046 kb fragment was used for microinjection. For linearized DNA the plasmid was digested with the *Bam* HI restriction enzyme. The location of the oligonucleotide primers used for PCR analysis, P1 and P2 are shown. The commercially available pGL2-Control Vector (Promega, WI, USA), containing the firefly *luciferase* gene under the control of the SV40 promotor and enhancer sequences on a pUC19 backbone (see Figure 2.1) was grown up and harvested using the Magic™ Maxiprep DNA Purification System (Promega) following the manufacturers instructions. For linearized DNA the plasmid was digested with the *Bam*H 1 restriction enzyme. The concentration of the DNA was determined using a spectrophotometer and the entire plasmid, in either the linear or circular form, was used for injection.

Two oligonucleotide primers 5'-GCGTGCTAGCTCGAGATCTG-3' and 5'-CGAAGTATTCCGCGTACGTG-3' (P1 and P2 respectively, in Fig 2.1) were designed, for PCR detection of the pGL2 transgene, using a computer program (PRIMER DESIGNER Version 2.0, Scientific and Educational Software). These primers generated a PCR product of 414 base pairs (bp).

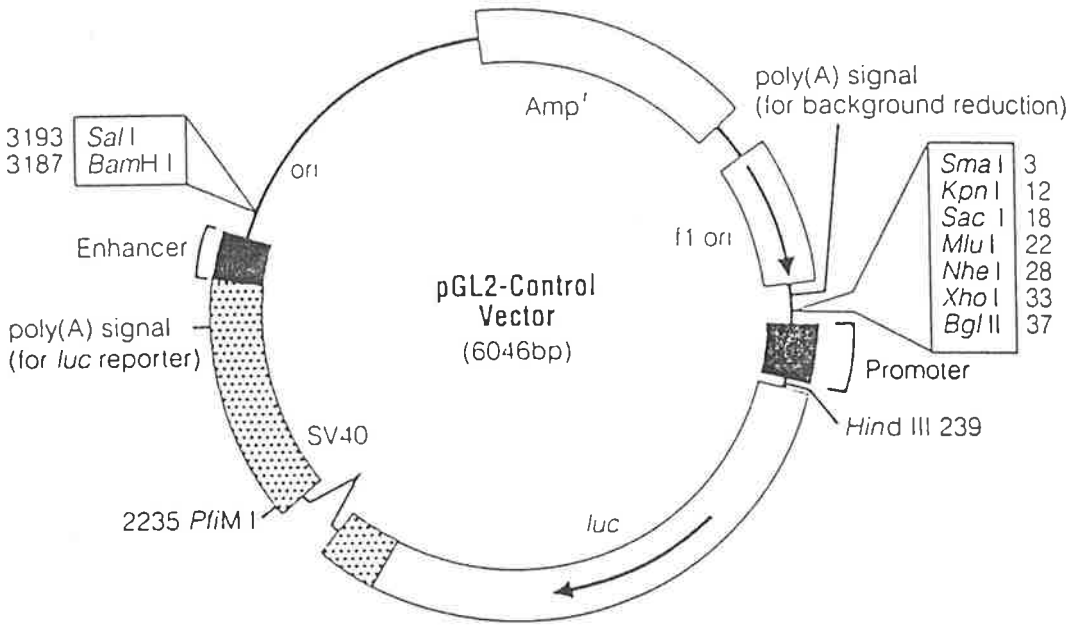


Figure 2.1 The restriction map and organization of the pGL2-control vector (Promega).

2.5.2 DNA Analysis

2.5.2.1 Collection of tissues

To extract genomic DNA, fish were anaesthetised in Benzocaine 25 mg/L and entire larvae or fin clips (taken from dorsal, ventral, caudal or anal fins) from adult fish were collected. Each fry or fin was rinsed in 3x Milli Q water, once in Tris-EDTA (10 mM Tris; 1 mM EDTA) (TE), placed individually in 0.5 ml microtubes, and snap frozen by plunging into liquid nitrogen (LN_2). Frozen samples were stored at -70°C until they were analysed.

Each sample was thawed and then digested in 25 µl (pooled batches of embryos were digested in an appropriate volume *ie* 100 µl) of Digest Buffer composed of 1x Amplification buffer supplemented with non-ionic detergent and proteinase K (67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)SO_4$; 0.45% Triton X-100; 200 µg/ml gelatin; 0.45% Nonidet P-40 and 60 µg/ml Proteinase K) at 55°C for 90 min and heated at 99°C for 15 min to inactivate the Proteinase K and any nucleases that might have been present. The digested mixture was centrifuged (3000 x *g* for 5 min) to pellet the undigested tissue and cell debris and the supernatant was recovered and stored at -70°C until use.

2.5.2.2 Purification of genomic DNA

Total nucleic acids were extracted by phenol-chloroform and ethanol precipitation from the supernatant recovered after digestion of pooled batches of larvae (section 2.5.2.1). Concentrations of genomic DNA resuspended in 1x TE were determined by measuring the UV absorption at 260 nm using a spectrophotometer.

2.5.2.3 Polymerase Chain Analysis (PCR)

Approximately 1 µg of purified genomic DNA or 1-2 µl of crude genomic extract was used for the PCR detection of the pGL2 transgene following

methods of Saiki, (1990), with modifications. The target DNA was placed in a 0.5 ml microfuge tube containing the PCR mixture (1x Amplification buffer; 2 mM $MgCl_2$; 200 μM each of dATP, dCTP, dGTP, dTTP; 2 μM each of primer and 1 unit of Taq Polymerase). The entire reaction mixture was overlaid with paraffin oil and amplified using a thermal cycler (FTS-1; Corbet Research, Australia). The reaction was carried out at 94°C for 1 min, 55°C for 1.5 min and 72°C for 1 min for 35x cycles with a 5 min initial 94°C denaturation step and a 10 min final 72°C elongation step. Then 15 μl of the amplified mixture was mixed with 3 μl of a 6x loading buffer and subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and photographed under UV light.

2.5.3 Transgene Expression Assays

2.5.3.1 Collection of tissues

Assays were routinely conducted on whole larvae, which were euthanised and washed 3x with Milli-Q water, prior to analysis.

2.5.3.2 Luciferase Assay

A commercially available Luciferase Assay System (E1500; Promega) was purchased in a kit form and assays were conducted following manufacturers instructions with minor modifications. Briefly, larvae were placed in 0.5 ml microfuge tubes in a 10 μl volume of 1x Cell Culture Lysis Reagent (125 mM Tris, pH 7.8 with H_3PO_4; 10 mM CDTA; 10 mM DTT; 50% glycerol; 5% Triton X-100). After allowing the samples to stand at RT for 15 min they were subjected to 3x cycles of freeze-thawing (LN_2:37°C). After a brief vortex the samples were subjected to another three cycles of freeze-thawing.

The reconstituted luciferase assay reagent (270 μM Coenzyme A [lithium salt]; 470 μM Luciferin; 530 μM ATP; 20 mM Tricine; 1.07 mM $(MgCO_3)_4$; $Mg(OH)_2 \cdot 5H_2O$; 2.67 mM $MgSO_4$; 0.1 mM EDTA; 33.3 mM DTT; final pH 7.8), was

thawed and allowed to reach RT. 90 µl of the assay reagent was added to each sample immediately prior to the light emission being measured using a scintillation counter (LS 3801; Beckman, USA) which had the coincidence circuit inactivated (Single Photon Counting mode). Scintillation counts were measured every 0.1 min over the first min and expressed as counts per minute (cpm).

2.5.3.3 β-Galactosidase Assay

β-Galactosidase expression was detected in larval fish employing an assay modified from that described by Tan (1991). Briefly: Collected larvae were washed 3x Phosphate Buffered Saline (PBS), and fixed with 1.25% Glutaraldehyde in PBS (pH 6.8) for 60 min, subsequently the samples were incubated overnight at 37°C in freshly prepared stain (0.1% 4 chloro-5 bromo-3 indolyl-*β*-D-galactopyranoside (X-gal; Sigma); 2 mM $MgCl_2$; 5 mM EGTA; 0.01% [w/v] sodium desoxycholate; 0.02% [w/v] Nonidet P-40; 5 mM $K_3Fe(CN_6)$; 5 mM $K_4Fe(CN)_6 \cdot 6H_2O$). [NB The X-gal stock consisted of a 4% solution made up in dimethylformamide.] Samples were subsequently washed 3x with PBS to remove excess stain, observed microscopically and photographed.

2.5.4 Karyotyping

Karyo-analysis of zebrafish embryos was performed following the methods of Streisinger *et al.*, (1981), with a few modifications (Kligerman and Bloom, 1977). Essentially; 48h zebrafish embryos, were dechorionated with fine forceps, and incubated in 100 µl of 10^{-2} M Colchicine (Sigma) solution in microfuge tubes on the warming tray for 2-3h. Colchicine prevents the movement of chromosomes by destroying the spindle fibres, thus arresting them at the metaphase plate. After dissecting away the yolk sacs the embryos were subjected to a hypotonic shock, this causes the chromosomes to swell, thereby enhancing visibility. 0.04

M Tri Sodium Citrate solution was used first at RT for 15 min, followed by a cold hypotonic shock at 4°C, for a further 15 min. Methanol:Acetic Acid at a ratio of 3:1 (v/v) was used as a fixative, and embryos were fixed for 60 min after which they were individually minced in 80 µl of 50% Acetic Acid, for 2 min prior to being repeatedly dropped onto and removed from slides at 48°C. The Acetic acid treatment induced the release of the chromosomes from the nuclear and cellular membranes. The slides were stained in 10% Giemsa for 10-15 min, washed with water to remove the excess stain, air dried, cleared in Savsolvent (Ajax Chemicals, NSW, Australia) and mounted with DPX mountant (BDH, Dorset, England). At least 5 spreads per slide were counted and/or photographed.

CHAPTER 3

SINGLE GENE MANIPULATION (TRANSGENESIS)

3.1 INTRODUCTION

The demonstration of the feasibility of applying transgenesis a decade ago to mammalian species (Gordon *et al.*, 1980; Palmiter *et al.*, 1982; Hammer *et al.*, 1985), attracted widespread interest particularly amongst livestock breeders interested in the potential of this revolutionary technology as a means of achieving rapid genetic improvement in animal genotypes. This led to the exploration of the potential of transgenesis to other vertebrates of research and commercial interest including amphibians (Bendig, 1981), and fish (Zhu *et al.*, 1985; Chourrout *et al.*, 1986a; Ozato *et al.*, 1986).

It was quickly recognised from early studies attempting to adapt the microinjection procedures used as a means of gene transfer in mammalian species that fish differed from mammals in several important ways:

1 Compared with mammals the pronuclei of most fish studied are not easily visualised (Maclean *et al.*, 1987; Chen and Powers, 1990), and researchers have accepted an approach based on injecting the transgenes into the cytoplasm. This exposes injected DNA to any nuclease activity present in the cytoplasm, increasing the possibility of degradation and/or rearrangement of injected sequences (Maclean *et al.*, 1987).

2 Compared with mammals even microinjection into the cytoplasm, is technically more difficult due to the tough chorion, which needs to either be removed by either chemical or mechanical methods, or methods to circumvent the problem need to be devised (reviewed by Maclean *et al.*, 1987; Ozato *et al.*, 1989; Chen and Powers, 1990).

3 Mosaicism, due to integration of the transgene into only a fraction of cells of the founder animals, is a problem commonly encountered in piscine transgenesis studies resulting in transgenic individuals transmitting the gene on to their progeny at frequencies below the 50% rate that can be expected from a heterozygous founder (Ozato *et al.*, 1986; Stuart *et al.*, 1988; Schneider *et al.*, 1989; Houdebine and Chourrout, 1991; Penman *et al.*, 1991; Inoue *et al.*, 1991; Culp *et al.*, 1991; Patil *et al.*, 1994). This is possibly exacerbated by the relatively short inter-cleavage interval exhibited by the embryos of most fish species in comparison to mammalian embryos.

4 One way to circumvent the problem is the novel method used effectively by Ozato *et al.*, (1986), for medaka *Oryzias latipes* where the transgene was injected into the oocyte nucleus prior to the germinal vesicle breakdown (GVBD). Unfortunately, this technique cannot be utilised for most fish species as *in vitro* maturation (IVM) of fish oocytes is in its infancy at present.

During the final maturation of *Xenopus levis* oocytes *in vivo*, genomic (chromosomal) DNA is protected from nuclease activity when GVBD occurs and the nuclear membranes disintegrate (Wyllie *et al.*, 1977). Foreign DNA sequences injected into oocytes at this stage should likewise be protected and gathered along with the genomic DNA (on activation of the oocyte) when the maternal chromosomes are gathered together and bound by the nuclear envelope to form the female pronucleus. This would permit integration at the earliest possible time, minimising the rate of mosaicism. However, ovulated zebrafish oocytes are prone to activate parthenogenetically, when diluted with any media or buffered salt solution preventing investigation of this hypothesis. There is no report in the literature of a diluent that has the ability to inhibit this auto-activation permitting the injection of zebrafish oocytes.

Most of the earlier work in this area (transgenesis), with some notable exceptions (Stuart *et al.*, 1988; Ozato *et al.*, 1986), has been based on studies with commercial species of fish (Zhu *et al.*, 1985; Chourrout *et al.*, 1986b; Maclean *et al.*, 1987; Brem *et al.*, 1988; McEvoy *et al.*, 1988). The long generation time of most of these species and requirements for expensive, secure, aquaculture facilities to rear experimental individuals has, we would argue, limited the rate of development of transgenesis technology in the piscine.

The aims of experiments reported in this chapter were therefore to identify a laboratory piscine model which would allow a higher rate of development in transgenic studies and utilise this species together with model reporter transgenes to explore the possibility of developing alternative gene transfer methods of research and commercial interest, such as sperm mediated transgenesis and oocyte injection.

3.2. TRANSGENESIS METHODS

3.2.1 Micromanipulation for Gene Transfer

3.2.1.1 Micromanipulation System for Gene Transfer

Microinjection was routinely performed using an Olympus (SZ 60) stereo dissecting microscope, fitted with two 3-dimensional Leitz micromanipulators. The injection pipette instrument holder was connected to a high pressure nitrogen gas cylinder *via* a 3-way valve gas flow switch (Festo Eng., Australia) using thick walled plastic tubing (Masterflex Tubing 6409-16;, Norton Performance Plastics, USA). The DNA was injected under pressure (220 kPa), when the bypass valve was depressed.

To control meniscus movement in the holding pipette, a gas tight (threaded plunger) 0.5 ml Hamilton syringe (Alltech Pty. Ltd., Australia) was connected to a Leitz instrument holder containing the holding pipette by teflon tubing. The

entire system was filled with silicon oil (200 fluid/20 centistokes; Dow Corning, USA).

3.2.1.2 Manipulation Pipettes

Microinjection and Holding pipettes were manufactured with glass capillary tubing (Clark Electromedical, UK). Capillary tubing of a 150 mm in length was pulled on a pipette puller (P-77B; Brown-Flaming, USA) to produce two pipettes with gradually tapered tips. Microinjection pipettes were manufactured from thin walled tubing (GC-100TF-15) to produce a closed tip of 0.1 μm outer diameter (OD). DNA was loaded into the pipette from the blunt end using capillary action. Opening of the microinjection pipettes to an OD of approximately 1 μm occurred by gently brushing the tip of the holding pipette. This procedure was conducted inside the microinjection chamber and observed through the microscope. Embryo holding pipettes were made from capillary tubing (GC100-15), that was not pulled as the OD of 1 mm is suitable, however the cut end of the tubing was heat polished to prevent damage to the embryos.

Using the microflame or microforge all pipettes were bent 100° twice at 6 mm and 14 mm intervals from the tip to facilitate entry into the manipulation chamber. Glass jars were used to store and protect pipettes until required.

3.2.2 Transgene Preparation

The transgene constructs were prepared as described in general methods (section 2.5.1). The DNA samples were diluted in phosphate buffered saline (PBS; Flow Laboratories, NSW, Australia) to the desired concentration and frozen (-20°C) in aliquots. Prior to microinjection, aliquots of DNA were centrifuged for 5 min at 3000 x *g*, at 4°C to remove particulate matter.

3.2.3 Microinjection of DNA

ZEBRA DANIO

One-cell zebrafish embryos produced by AI (as described in section 2.3.1.2) were placed in the lid of a 36 mm disposable plastic culture dish filled with filtered tank water, which served as the micromanipulation chamber.

The metaphase II oocytes were microinjected in 50 µl drops of sperm suspension placed in the manipulation chamber. The eggs and pipettes were brought into focus at the optimal magnification. The embryos or oocytes were held with the holding pipette and the gas pressure tap was then depressed to inject approximately 600 picolitres (pl) of DNA solution into the cytoplasm. Successful microinjection was confirmed by the darkening of the cytoplasm where the DNA solution is released. Oocytes were subsequently fertilized and embryos were returned to dishes on the warming tray at 26°C to commence development.

MEDAKA

Microinjection into the germinal vesicle (GV) of oocytes was performed according to the method of Ozato *et al.*, (1986). Essentially, medaka oocytes were injected prior to the germinal vesicle break down (GVBD) stage in E199 culture media drops (for the preparation of media see section 2.1.3), with the DNA being injected into the GV of the oocyte which is clearly visible at the animal pole. Subsequently the injected oocytes were returned to 4-well culture plates on the warming tray and maintained at 26°C to allow final maturation and ovulation to be completed, after which they are inseminated with a sperm suspension, and allowed to develop.

3.2.4 Evaluation of Gene Transfer

Tissues were collected and presence of the transgene in embryos and adult fish was assessed by the Polymerase Chain Reaction (PCR) technique as described in general methods (section 2.5.2).

Expression assays of the transgenes employed were carried out using methods described in general methods (section 2.5.3).

3.3. STATISTICAL ANALYSES

Group data was analysed using ANOVA and individual values were compared using the Chi-Square test. For experiments 3.4.1 and 3.4.2, (Table 3.1 and Table 3.3), individuals that displayed values more than 2 standard deviations from the mean of control values were considered to be positive. Data in Table 3.6 was analysed using ANOVA, extreme data was tested using the Smirnoff's test to determine whether it could be ignored. The data was subsequently tested using *T-test*.

3.4 EXPERIMENTAL

3.4.1 Comparison of Sperm Mediated Transfer of Circular and Linear Constructs in the Zebrafish *Danio rerio*

Aim: To determine the utility of the *luciferase* gene as a reporter gene for transgenesis, including sperm mediated transfer, in zebrafish.

3.4.1.1 Outline

Zebrafish gametes were collected for AI (section 2.3.1.2). Aliquots of sperm diluted in HBSS at a final concentration of 50 ±5 $x10^6$ sperm/ml were incubated with the pGL2 construct which contained the *luciferase (luc)* gene driven by an SV40 promoter and enhancer region (Figure 2.1), in either the circular or

linearised form (for details of transgene preparation see section 2.5.1), at a final DNA concentration of 200 μg/ml, for 30 min at RT. Batches of zebrafish oocytes collected from ovulating females were fertilised with sperm belonging to either circular or linearised DNA treated sperm group, along with a corresponding control group of oocytes that were fertilised with an aliquot of sperm that was not exposed to DNA. Eggs were rinsed after a period of 15 min to remove excess sperm, and maintained at 26°C. The number of embryos that were successfully fertilised, was recorded for each group.

Embryos belonging to both the treatment groups (linear and circular plasmid) and the control (no DNA) group, were then analysed for expression of the transgene at 24h pf, using a luciferase (LUC) assay (section 2.5.3.2). Preliminary studies had indicated this was a convenient stage to detect LUC activity, as it was the latest stage for performing the assay prior to the onset of pigmentation, which decreased the sensitivity of the LUC assay. The number of individuals in each group that expressed LUC activity was recorded. Some embryos from experiments where sperm was incubated with pGL2 DNA in the linear form were reared, along with the corresponding controls, to maturity. Fins collected from the resultant adult fish (F0) under anaesthesia were analysed by polymerase chain reaction (PCR) for presence of the transgene. Fin positive F0 individuals were then mated with wild type (non-transgenic) fish to determine the heritability of the transgene by examining the progeny (F1) for presence of the transgene. This experiment was performed as a series of four replicates.

3.4.1.2 Results

Zebrafish sperm incubated with DNA were found to be capable of transferring the transgene to resulting embryos and the rates of transfer and levels of expression of the transgene, analysed in 24h embryos, was the same (14%) for sperm exposed to the transgene in either the circular or the linearised form (Table 3.1).

Sperm mediated transfer of transgenes into zebrafish embryos was, however, not as efficient as was seen for medaka (experiments reported later in this chapter, section 3.4.5) with gene transfer rates of 14 and 36% achieved respectively (Table 3.1 and Table 3.8), despite the sperm being exposed to much higher concentrations of DNA (200 and 10 μg/ml, respectively), however large numbers of offspring can be generated due to the favourable fecundity displayed by the zebrafish. The transgenes used for the these studies were different, but preliminary investigations with zebrafish using the same construct used for medaka (*lacZ*) gave similar results to those reported above (data not shown). The reduced efficiency of gene transfer achieved for zebrafish is consistent with results, reported by Stuart *et al.*, (1988; 1990), when transgenes were microinjected into fertilised zebrafish embryos.

Of interest was the fact that as early as 12h pf, LUC expression in experimental embryos displayed a trend toward significance (data not shown), however, fully formed larvae that have yet to undergo pigmentation (24h) were at the optimal stage to be assayed for LUC activity.

The gene transfer rate determined in adult F0 fish by PCR analysis of fin clips was not significantly different from that estimated for embryos by LUC assay (13 and 14% respectively). That only a small proportion (2/5) of the fin positive F0 fish tested were germline transformants was an unexpected result, moreso as even the two germline positive F0 fish only transmitted the transgene to their progeny (F1) at low frequencies of 10-18% (Table 3.2), suggesting transgene mosaicism in the F0.

3.4.2 Patterns of Expression of Luciferase , Exhibited by Zebrafish Embryos, During Early Development

Aim: To study the expression profiles of the *luc* reporter gene microinjected into zebrafish zygotes at the 1-cell stage.

3.4.2.1 Outline

Artificially inseminated (synchronously cleaving) zebrafish zygotes were injected, with approximately 600 pl of pGL2 at a final concentration of 100 μg/ml (which approximates to about 9 $x10^6$ copies of the gene), prior to the first cleavage division. Embryos were rinsed in tank water and maintained at 26°C. The number of embryos that survived microinjection and commenced normal cleavage was recorded. Batches of embryos resulting from zygote injection, were collected, with the appropriate controls, at pre-determined time points, ranging from 2 to 168h pf (Note: As some eggs were maintained at lower temperatures, with the intention of delaying cleavage to facilitate microinjection, Time 0 was calculated back based on the time at which the embryos reached the 4-cell stage of development).

Embryos in individual 0.5 ml microtubes, were snap frozen by plunging in LN_2 (as described section 2.5.3.1) and stored dry at -20°C until they were assayed. Three replicated sets of observations were conducted.

3.4.2.2 Results

Expression of the *luc* gene was detected in all the microinjected embryos as early as 5h pf (blastula stage). A dramatic increase in LUC expression was observed between 11 and 15h of development (somitogenesis) which peaked by 24h and was still elevated in 72h embryos. By 120h pf the expression levels had dropped considerably (but were still significantly elevated compared to controls), and by 168h pf they had returned to control values (Table 3.3; Figure 3.1).

Deformed embryos appearing in the initial 24h resulting from the microinjection groups, consistently displayed higher levels of LUC expression than corresponding microinjected embryos that developed normally, suggesting that the deformities are a result of either; integration of a large number of copies, or due to high levels of expression of the gene, rather than as a result of the microinjection procedure itself (Table 3.3; Figure 3.1).

3.4.3 Inhibition of Parthenogenetic Activation of Ovulated Zebrafish Oocytes

Aim: To use varying concentrations of sperm suspension in an attempt to arrest the parthenogenetic activation characteristic of zebrafish ovulated (metaphase II) oocytes.

3.4.3.1 Outline

Sperm from a number of spermiating males was pooled in 150 µl cold HBSS, to give a final concentration of 50 ±5 $x10^6$ sperm/ml (the concentration of spermatozoa was determined using a haemocytometer). The sperm suspension was serially diluted in HBSS and aliquots at the resulting dilutions were used to inseminate batches of ovulated oocytes which were held at RT for 30 min and subsequently activated with tank water. Groups of oocytes, at each dilution of the sperm suspension were inseminated and activated immediately, serving as appropriate controls.

To examine whether seminal fluid alone could arrest the parthenogenetic activation of oocytes, an aliquot of the undiluted sperm suspension was subjected to centrifugation to pellet the sperm and freshly ovulated oocytes were exposed to the supernatant, containing seminal fluid devoid of spermatozoa, for a period of 30 min. A fresh aliquot of sperm was then added to this batch prior to activation of the oocytes.

After effecting fertilisation, the eggs were washed to remove excess sperm and maintained at 26°C. The number of eggs assigned to each treatment group, and the number of embryos that successfully underwent cleavage, was recorded. The occurrence of deformities was also noted. This study was conducted as a series of three replicates.

3.4.3.2 Results

Fertilisation rates for control groups of oocytes at the serial dilutions tested (50-1.6 $x10^6$ sperm/ml) were not significantly different from one another or from the groups of oocytes inseminated at the highest concentration of sperm (50 $x10^6$ sperm/ml) and activated after 30 min (Table 3.4), however when oocytes were inseminated with serial dilutions of sperm suspension, and activation was effected after a 30 min delay, the rate of successful fertilisation was proportional to the concentration of sperm (Figure 3.2). A dramatic decrease ($P<0.001$) in delayed fertilisation rates was noted when the sperm concentration decreased from 25 to 12.5 $x10^6$ sperm/ml (77 and 17%, respectively).

It was further determined that factors present in seminal fluid, and not spermatozoa, are responsible for the phenomenon reported, as the groups of oocytes inseminated with sperm suspensions devoid of spermatozoa, displayed the ability to inhibit auto-activation of oocytes with no adverse effects on subsequent fertilisability of the oocytes. The fertilisation rates of 92% (24/26) achieved for this group were not significantly different from the control group (94%).

The marked decrease, in delayed fertilisation rates of oocytes, achieved when the sperm count drops below 25 $x10^6$ sperm/ml, did not pose a serious technical problem, as sperm suspensions used routinely for AI, contain spermatozoa at concentrations of between 50 and 100 $x10^6$ sperm/ml.

Seminal fluid obtained from spermiating male zebrafish was capable, at extremely low concentrations, of inhibiting spontaneous activation of ovulated zebrafish oocytes, as we routinely collected 2-3 µl milt (semen) per male, and typically the milt from 4-5 males was diluted in 150 µl HBSS (~10 fold dilution), to provide the sperm suspension for insemination.

3.4.4 Oocyte Injection as an Efficient Route to Producing Transgenic Zebrafish.

Aim: To determine whether transgenes microinjected into ovulated (metaphase II) oocytes as compared to zygotes (1-cell embryos); can survive, integrate and be transmitted, *via* the germline, to subsequent generations. Also, whether microinjection at this early stage reduces the degree of transgene mosaicism that has been reported for cytoplasmic injection of zygotes.

3.4.4.1 Outline

Batches of either, fertilised 1-cell embryos or ovulated metaphase II oocytes which were inseminated (but not activated) with a sperm suspension, were injected with approximately 600 pl of pGL2 at a final concentration of 100 µg/ml. The injected oocytes were subsequently activated. The fertilised embryos, belonging to both injection groups, were then washed to remove excess sperm and maintained at 26°C. Batches of uninjected eggs from both groups were maintained as controls.

Five developmentally normal embryos per replicate for both injection groups along with uninjected control embryos were collected and assayed for LUC activity at 24h pf. The remaining embryos were reared to maturity. Embryos were examined daily and the number that were developing normally or abnormally noted.

Fin clips were collected from the adult F0 fish and the presence of the transgene determined by PCR analysis (Plate 3.1). F0 fish identified as being fin positive by PCR were mated with wild type (non injected) individuals. To identify which F0 individuals were germline transgenics, genomic DNA from the F1 progeny of transgenic founders was extracted from pooled batches of 8-10 larvae each (24-72h pf), and ~1 μg of this was used as the target DNA for PCR analysis. At least 30 F1 larvae from each spawning transgenic F0 were analysed. Once a germline positive F0 fish was identified, PCR analysis on individual F1 embryos produced by that founder was carried out in order to determine the transmission rate. 30 24h F1 embryos resulting from the first identified germline positive F0 fish (M2) were assayed for LUC expression to determine expression of the transgene. This study was conducted as a series of six replicated observations.

3.4.4.2 Results

Microinjection compromises the survival of embryos, as the survival to 24h, of embryos belonging to both the injection (zygote and oocyte) groups, was significantly ($P<0.001$) lower than their respective controls (81 *v/s* 97% and 77 *v/s* 94%, respectively). The rates of survival of embryos in the two injection groups were not significantly different from each other, however, the rate of deformity observed for embryos in the oocyte injection groups was higher ($P<0.05$) than that noted for the zygote injected group (48 and 38% respectively, Table 3.5).

Also the percentage of oocyte-injected embryos (64%) that successfully commenced cell division was significantly lower ($P<0.001$), than either of the controls groups (oocyte control and zygote control, 88 and 90% respectively) and the zygote injected group (90%). The control groups were not significantly different from one another in terms of the percentage of embryos that either underwent cleavage or survived to 24h pf (Table 3.5).

24h embryos resulting from oocyte injection displayed considerably higher levels of expression of pGL2 than embryos resulting from zygote injection, and assayed at the same stage of development ($P<0.0001$) (Table 3.6; Figure 3.3).

The incidence of transgenesis in adult F0 zebrafish was greatly influenced by the stage at which microinjection was performed, with the frequency of fin positive F0 adults resulting from the oocyte injection group being double that recorded for the zygote injection group (47 and 25%, respectively; Table 3.7).

Most of the fin positive F0 adults tested in the oocyte injection group (6/7) and a large proportion in the zygote injection group (6/9) proved to be germline transformants, making fin analysis a convenient screen for identifying putative germ positive F0 fish.

Germline positive F0 adults arising from oocyte injection transmitted the transgene at a significantly higher ($P< 0.001$) frequency to their progeny than those resulting from zygote injection (44 and 12% respectively; Table 3.7). 47% (14/30) of the F1 embryos from the M2 germline positive F0 expressed LUC and this frequency was not significantly different to that obtained by DNA analysis of siblings (from the same F0; Table 3.7).

3.4.5 Sperm Mediated Transfer of Novel Genes in Medaka *Oryzias latipes*

Aim: To evaluate the efficacy of sperm as a vehicle to effect the transfer of foreign genes into the medaka embryo.

3.4.5.1 Outline

Medaka spermatozoa were collected as described in general methods (section 2.3.2.2). Suspensions with sperm concentrations of approximately 5 $x10^7$ sperm/ml were split into two aliquots. One of these aliquots was then

incubated at RT for 30 min, with either a RSV-*lacZ* construct which consisted of the β-Galactosidase gene driven by the long terminal repeat (LTR) of the *Rouse Sarcoma Virus* promotor, or the pHMPG.4 construct which contained the porcine growth hormone gene controlled by the human metallothionein IIA promotor (for details of transgene constructs see section 2.5.1), at a final DNA concentration of 10 μg/ml. The second aliquot of sperm suspension was also incubated under the same conditions but in the absence of the DNA construct, and used as a negative control. Following incubation, the sperm suspensions were used to fertilise batches of freshly ovulated oocytes. After a period of 15 min the fertilised eggs were rinsed to remove excess sperm, and maintained at 26°C. For comparison, the RSV-*lacZ* construct, at a concentration of 10 μg/ml was injected into the 4C nucleus of pre-GVBD (germinal vesicle break down) stage medaka oocytes. The oocytes were then subject to *in vitro* maturation (IVM), and AI procedures (as described in general methods, section 2.3.2.2 and 2.3.2.3, respectively) to serve as a positive control. A further group of uninjected oocytes that underwent IVM were also fertilised to serve as an untreated control group. Oocytes that underwent successful IVM, AI and embryonic development were noted and their numbers recorded. Putative transgenic embryos (Day 10-12) for the pHMPG.4 and *lac Z* constructs, were tested for either presence or expression of the transgene by either PCR analysis or β-Galactosidase assay (section 2.5.3.3), respectively. This study was conducted as a series of three replicates.

3.4.5.2 Results

The frequency of transfer of transgenes into medaka by either sperm mediated transfer using the two different gene constructs tested or microinjection into the GV nucleus of pre-GVBD oocytes, was not significantly different, ranging from 33-38% (Table 3.8.), average 36%.

Uninjected oocytes that were collected at a post ovulatory stage for AI, or at the GV stage and induced to undergo IVM and subsequent AI, survived to the 24h stage at rates that were not significantly different (85 and 73%, respectively), however embryos belonging to the sperm mediated experimental groups displayed significantly ($P<0.01$) lower survival to the same stage than control embryos and embryos in the GV injection group showed the lowest survival compared to both the control groups ($P<0.001$) and the sperm mediated transfer groups ($P<0.01$; Table 3.8.). β-Galactosidase was expressed in the otoliths and to a lesser extent along the lateral line (between the somites) in transgenic fish. However, the gut of control fish displayed endogenous β-Galactosidase activity (See plate 3.2), presumably due to bacteria present, which could not be destroyed even with high doses of antibiotics.

3.5 DISCUSSION

Transgenic techniques have only recently been applied to fish species but the results are very encouraging (Chen and Powers, 1990). The major problems associated with the transfer of transgenesis technology to fish, highlighted by the early studies, are the long generation time inherent to most commercial species, therefore the need for a suitable model species, and also the need for efficient mass gene transfer techniques coupled to methods that permit convenient rapid screening of large numbers of putative transgenics (Chen and Powers, 1990). The incidence of transgene mosaicism in F0 fish which appears to be a feature of fish transgenesis (Ozato *et al.*, 1986; Stuart *et al.*, 1988, 1990; Schneider *et al.*, 1989; Houdebine and Chourrout, 1991; Penman *et al.*, 1991; Culp *et al.*, 1993; Patil *et al.*, 1994) and the lack of expression of the transgene in some studies (Stuart *et al.*, 1988; Khoo *et al.*, 1992; Culp *et al.*, 1993; Patil *et al.*, 1994) need to be addressed.

Transgenesis has been previously achieved with the medaka, through injection into the GV of oocytes prior to fertilisation (Ozato *et al.*, 1986; 1989). In the present study we were able to obtain a similar transgenesis rate using either sperm as vectors to transfer the transgenes to the resulting embryo or by microinjection of transgenes into the GV of maturing oocytes, (36 and 33% respectively). The results achieved in this series of experiments endorse the use of medaka, as a model for transgenic studies; unfortunately some limitations have also been highlighted:

1 The reduced tolerance of medaka to survive long periods of tropical temperatures (26-28°C) limit the use of this species in Australia and other tropical climatic zones during summer months, unless a suitable low temperature housing facility is available.

2 Medaka have relatively low fecundity in comparison with most other species of fish, in addition the broodstock have to be sacrificed to collect gametes.

3 Additionally due to the stringent quarantine regulations existing in Australia the availability of varied strains of medaka is restricted.

4 Due to the high fecundity characteristics of most species of fish, the ability to treat large numbers of eggs with DNA accentuated the need for a suitable reporter gene, that can be detected cheaply, preferably by an expression detection system that would circumvent the need for DNA analysis in preliminary studies.

5 Genes such as *lac Z* coding for enzymes such as β-Galactosidase which have a convenient assay are desirable however, we report an unforeseen problem with background activity of the above mentioned enzyme (which is of bacterial origin), presumably due to the bacteria present in the gut, which are impractical to eliminate. Other genes of bacterial origin like *cat* were rejected for the same reasons.

For the above mentioned reasons the focus of these studies were transferred to the zebra danio, a member of the Cyprinid family (which includes species of commercial interest such as the carps).

Transgenes including *luc* sequences have been previously used in fish (Tamiya *et al.*, 1990; Gibbs *et al.*, 1991; Patil *et al.*, 1994). The current study confirmed the value of *luc* as a suitable reporter gene the expression of which can be examined by an assay that is cheap, rapid to run and extremely sensitive. More importantly the problems of background expression observed with reporter genes of bacterial origin were eliminated.

Khoo *et al.*, (1992), reported the use of sperm as vectors to enable transfer of foreign genes into zebrafish embryos at high rates (37.5 and 23.3% respectively) for linear and circular plasmids, however they failed to detect expression of the transgene, despite having used a construct (pUSVCAT) that had been previously shown to be expressed when microinjected into zebrafish zygotes (Stuart *et al.*, 1990). This study, using the pGL2 construct for sperm mediated transgenesis, demonstrated successful transfer (determined by expression assays) of the transgene into zebrafish embryos at a rate of 14% (14/101), and expression of the novel gene was detected in 24h embryos. The gene was detected in the fin of adult founders at 3 months of age at a similar frequency of 13% (8/64). No significant difference in transfer rates was noted for circular or linear plasmids.

Surprisingly only a few of the fin positive F0 transgenic fish generated by sperm mediated transgenesis transmitted the transgene to their F1 progeny and that too at low frequencies (10-18%) which suggest that despite the transgene being carried to the embryo by the spermatozoa it did not reduce the incidence of mosaicism. Khoo *et al.*, (1992) reported a similar finding. As our interest lay

in producing stable germline transformants, linear constructs were used for further investigation.

The phenomenon of extrachromosomal replication of DNA sequences injected into early zebrafish embryos has been documented using DNA analysis studies, (Stuart *et al.*, 1988), and the present study examined this occurrence based on studies investigating the expression of a *luc* construct microinjected into zygotes, which demonstrated that LUC levels can be detected in embryos as early as the blastula stage (5h), and displays a dramatic increase in 15h embryos, peaking at 24h and subsequently dropping back to control levels by 168h of development.

Zebrafish oocytes are prone to undergo spontaneous activation on dilution. There is no report in the literature of a suitable diluent that has the ability to inhibit this parthenogenetic development. A significant finding in the present study was the demonstration that seminal fluid (diluted in HBSS) had the ability to inhibit spontaneous activation of zebrafish oocytes without compromising their viability, thus facilitating microinjection of transgenes at this stage. A dramatic loss of viability of oocytes held in seminal fluid was observed when suspensions with sperm concentrations below 25 $x10^6$ sperm/ml were used to delay activation but this did not prove a problem in practice as sperm concentrations normally used for insemination (50 - 100 $x10^6$ sperm/ml) were adequate to achieve satisfactory fertilisation after microinjection.

In previous studies, with fish species in general and zebrafish in particular (Stuart *et al.*, 1988; 1990; Culp *et al.*, 1991; Patil *et al.*, 1994), a high incidence of transgene mosaicism has been consistently recorded with the result that the transmission rates of the transgene by germline transformed F0 fish to F1 progeny varying from 6-36%. By comparison transgenes injected into oocytes,

not only survive and are present in the fins of founder fish at a high rate (47%) but are integrated into the germline of founders at a very high frequency (ave. 44%), when compared to conventional zygote injection suggesting hemizygous integration of the transgene in the founders, or a significant reduction in the incidence of mosaicism. Expression of the pGL2 transgene was detected in the one F1 line (M2) tested with the frequency of expressers not significantly different to that assessed by DNA analysis of siblings.

During the preparation of this thesis some F1 embryos resulting from the M2 founder fish were reared and 48% (11/23) of adult F1 fish retained the transgene (as assessed by PCR analysis of fin clips; data not shown) providing further evidence of hemizygous integration of the transgene.

The high deformity rate noted in the transgenic group, we suspect, is caused as a result of integration of a large number of copies of the gene, or high levels of expression of the gene, rather than due to any physical damage caused by microinjection.

In conclusion these studies highlight the importance of model species in the development of robust technologies, and establish the suitability of the zebra danio with regard to all facets of gene manipulation, including sperm mediated transgenesis, and oocyte injection. More importantly, the ability to inhibit parthenogenetic activation of oocytes, enabling the microinjection of transgenes at this stage, resulting in the production of hemizygous transgenic zebrafish at extremely high frequencies, has been demonstrated.

Table 3.1 Sperm mediated transgenesis in zebrafish embryos.
Comparison of the effect of Linearised and Circular forms of the pGL2 transgene on survival and transgenesis rate in early zebrafish embryos. Zebrafish sperm was incubated with either the linearised or circular forms of the pGL2 construct, prior to fertilisation. Embryos belonging to each of the experimental and control groups were assayed for luciferase (LUC) expression at 24h. For detailed experiment design see text.

Treatment	Treated	Embryo Survival at 24h		Analysed	Positives	
Group	n	N*	A*	n	n	%
Linear	296	260	2	66	9	14
Circular	116	52	1	35	5	14
Control	174	128	0	34	0	-

* morphology of embryos N = normal; A = abnormal.

Table 3.2 Incidence of transgenesis in F0 and F1 generations of zebrafish resulting from sperm mediated transgenesis.

Larvae produced by artificial insemination (AI) of oocytes with sperm incubated with the linearized form of the pGL2 transgene were reared to maturity. Fins collected from F0 founder fish were analysed by PCR analysis for the presence of the transgene. F1 fish produced by breeding the fin positive F0 with non transgenic fish, were analysed by PCR to determine the transmission rates of the germline positive F0.

Treatment Group	Analysed F0 n	Fin positive F0 n	Fin positive F0 %	F0 line Tested	F1 positive fish No.	F1 positive fish %
Sperm Mediated	64	8	**13**	S 31	0/20	-
				S 32	0/25	-
				S 33	2/20	**10**
				S 35	0/23	-
				S 36	4/22	**18**
	Germline data				6/42	**14**

Table 3.3 Sequential expression of luciferase (LUC) by zebrafish embryos microinjected with the pGL2 transgene.

Zebrafish embryos microinjected at the zygote (1-cell) stage with the pGL2 transgene were collected at the specified times post fertilisation and assayed for luciferase expression. LUC activity is expressed as CPM $x10^3$ (counts per minute) and each data point represents values for one embryo. Mean values were calculated using data from normal embryos only. Control values are representative of the control group.

Embryo Number	Stage of embryo in hours post fertilisation								
	2h	5h	8h	11h	15h	24h	72h	120h	168h
1	15.6	51	184	190	420	428	782	62.8	26.1
2	15.3	95	349	1559	564	1140	253	29.5	27.2
3	15.4	1489	112	742	970	2424	3374	38.9	25.0
4	15.4	35	35	310	2025	1230	4141	46.1	29.1
5	15.9	123	183	132	1672	5446	1960	39.3	25.7
6	15.6	33	1825	685	3767	1356	503	31.1	25.0
7	15.3	93	375	310	738	469	1011	24.8	23.8
8	15.4	25	459	279	6668*	652	265	37.4	28.9
9	16.3	47	180	3023*	1089*	3088	1574*	93.6	26.4
10	14.5	143	114	770*	5735*	9681*	384*	26.1	25.7
11							335*	42.5	26.5
12								30.1	
13								99.7*	
14								2349.9*	
Mean	15.5	213.4	381.6	525.9	1450.9	1803.7	1536.1	41.9	26.3
Control	15.6	18.9	17.1	15.3	16.3	16.4	18.3	19.8	22.4

* Denotes deformed embryo.

Table 3.4 Effect of sperm concentration on inhibition of parthenogenetic activation of ovulated zebrafish oocytes.

Ovulated (metaphase II) oocytes were placed in serially diluted aliquots of sperm suspension in parallel. Experimental batches of oocytes were held at room temperature for 30 min prior to the gametes being activated (DELAYED group) and control batches of oocytes were placed in sperm suspensions at the various sperm concentrations and fertilised immediately.

Sperm conc. $x10^6$ sp/ml	Percent survival at 24h / (No. survived/No. treated)	
	CONTROL	DELAYED ACTIVATION
50.0	**94 %**[a] (61/65)	**89 %** [a] (32/36)
25.0	**92 %**[a] (44/48)	**77 %** [a] (17/22)
12.5	**93 %** [a] (27/29)	**17 %**[b] (7/41)
6.3	**92 %** [a] (36/39)	**11** (4/37)
3.1	**92 %** [a] (36/39)	**2** (1/43)
1.6	**97 %** [a] (36/37)	**0** (0/35)
0.0	**0** (0/41)	**0** (0/48)

Values within the table with different superscripts differ significantly **(P<0.001).**

Table 3.5 Fertilisability normality and survival of embryos following microinjection of transgenes at the ovulated oocyte (metaphase II) and zygote (1-cell) stages.

Zebrafish embryos resulting from microinjection of pGL2 at either the oocyte or the zygote stage along with appropriate controls were cultured (oocytes were fertilised prior to culture) and records of embryo development were maintained for each group. Embryos belonging to the control groups were not microinjected.

Treatment Group	Treated	Cleaving		Survival of cleaving embryos at 24h		% Survivors
	n	n	%	n	%	deformed
Oocyte injected	283	181	**63.9[a]**	139	**76.8[a]**	**48.2[c]**
Oocyte control	118	104	**88.1[b]**	98	**94.2[b]**	**4.1**
Zygote injected	551	493	**89.5[b]**	401	**81.3[a]**	**37.7[d]**
Zygote control	558	500	**89.6[b]**	484	**96.8[b]**	-

Values within columns with different superscripts differ significantly, **[a-b] = (P< 0.001); [c-d] = (P< 0.05).**

Table 3.6 Comparative levels of expression of the pGL2 transgene by zebrafish embryos at 24h following microinjection into either metaphase II oocytes or zygotes.

Normal embryos belonging to both of the injection groups along with uninjected control embryos were sampled at 24h post fertilisation and assayed for luciferase activity using a scintillation counter. Counts per minute $x10^3$ were averaged over the first minute. Each data point represents values for one embryo.

Sample No	CONTROL	Stage of Microinjection	
		OOCYTE	ZYGOTE
1	22.8	18866	314
2	22.6	12040	6424
3	17.4	14487	603
4	17.0	5847	4794
5	18.4	9414	1838
6	19.8	20329	502
7	18.6	23327	7485
8	20.1	19950	2367
9	18.0	14872	4339
10	21.1	16111	428
11	23.3	10940	1140
12	19.7	18742	2424
13	18.9	9820	1230
14	19.7	19201	5446
15	21.9	17589	1356
16	17.5	21582	469
17	18.6	16138	652
18	19.2	19483	3088
19	22.2	9940	4678
20	17.5	13590	3289
21	18.9	19230	780
22	19.3	17423	998
23	18.2	10899	2398
24	17.1	8996	4501
25	19.9	15234	4867
26	23.0	16672	543
27	21.6	17986	652
28	21.0	10783	967
29	20.7	12567	3561
30	18.7	10780	2390
Mean	19.8^a	15095^b	2484^c

Values within the table with different superscripts differ significantly **(P<0.0001).**

Table 3.7 Incidence of transgenesis in F0 and F1 generations of zebrafish resulting from zygote and oocyte injection.

Embryos resulting from microinjection of pGL2 at either the zygote or metaphase II oocyte stages were reared to maturity. Fins were collected from F0 founder fish under anaesthesia and presence of the transgene was detected by PCR analysis. Transgenic F0 fish were bred with non-transgenic fish and the resulting F1 progeny were analysed individually by PCR to determine the transmission rates of the germline positive F0.

Treatment Group	Analysed F0 n	Fin positive F0 n	Fin positive F0 %	F0 line Tested	F1 positive fish No.	F1 positive fish %
Zygote Inject	61	15	**25[a]**	M21	0/30	-
				M22	0/30	-
				F23	3/29	**10**
				F24	3/17	**18**
				M25	3/24	**13**
				M28	3/18	**17**
				M29	0/20	-
				M30	3/30	**10**
				M31	2/30	**7**
	Germline Data				17/148	**12[c]**
Oocyte Inject	43	20	**47[b]**	M1	0/30	-
				M2	12/25	**48**
				F3	10/20	**50**
				M5	7/13	**54**
				F6	10/36	**28**
				F7	11/20	**55**
				M8	9/20	**45**
	Germline Data				59/134	**44[d]**

Values within columns with different superscripts differ significantly, **[a-b] = (P<0.025); [c-d] = (P<0.001).**

Table 3.8. Introduction of transgenes into medaka embryos; survival and transgenesis rate.
Gene transfer was effected in medaka either by microinjection into the germinal vesicle (GV) of maturing oocytes or using medaka sperm as vectors to transfer the genes into resulting embryos. Embryos resulting from the experimental and control groups were analysed for either expression or presence of the transgene at Day 7 of embryonic development.

Treatment Group	Construct	Treated	Survival at 24h		Analysed	Positive		Analysis Method
		n	n	%	n	n	%	
Sperm Mediated	pHMPG.4	90	48	**53[a]**	24	8	**33**	PCR
Sperm Mediated	RSV β-Gal	93	52	**56[a]**	37	14	**38**	β-Gal Assay
Control AI*		101	74	**73[b]**	17	0	-	
GV Inject & IVM*	RSV β-Gal	52	16	**31[c]**	12	4	**33**	β-Gal Assay
Control IVM*		40	34	**85[b]**	17	0	-	

*Abbreviations: **GV Inject** = microinjection into the germinal vesicle of the maturing oocyte; **IVM** = *in vitro* maturation; **AI** = artificial insemination.
Values within columns with different superscripts differ significantly, [a-b, a-c] = **(P< 0.01)**; [b-c] = **(P<0.001)**..

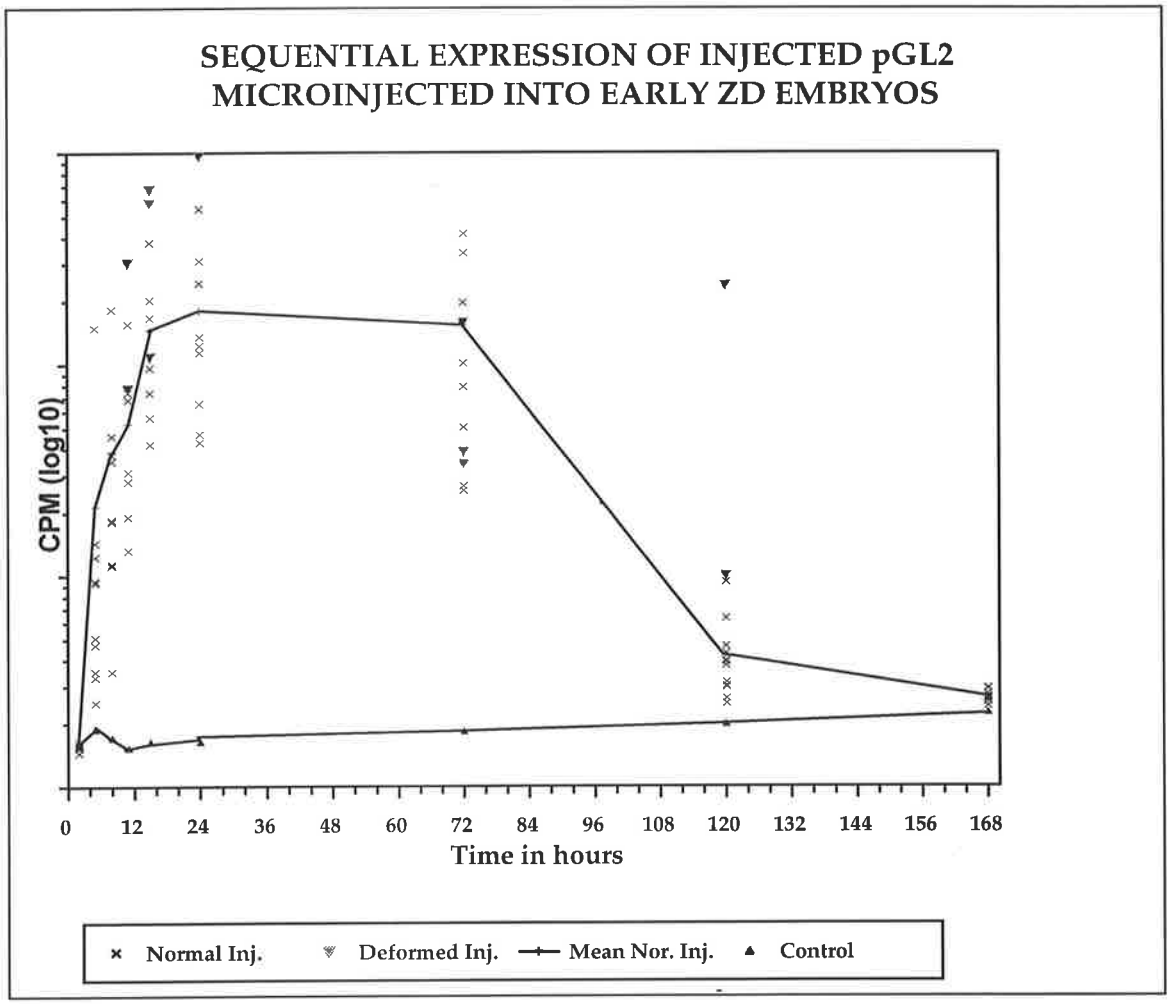


Figure 3.1 Sequential expression of luciferase by zebrafish embryos.
Zebrafish zygotes microinjected with the pGL2 construct were collected at varying times during development (2, 5, 8, 11, 15, 24, 72, 120, and 168h pf) and assayed for luciferase (LUC) expression. Expression of LUC was measured using a scintillation counter (CPM) and expressed logarithmically (log 10). Values for individual embryos are plotted. Data was sourced from Table 3.3.

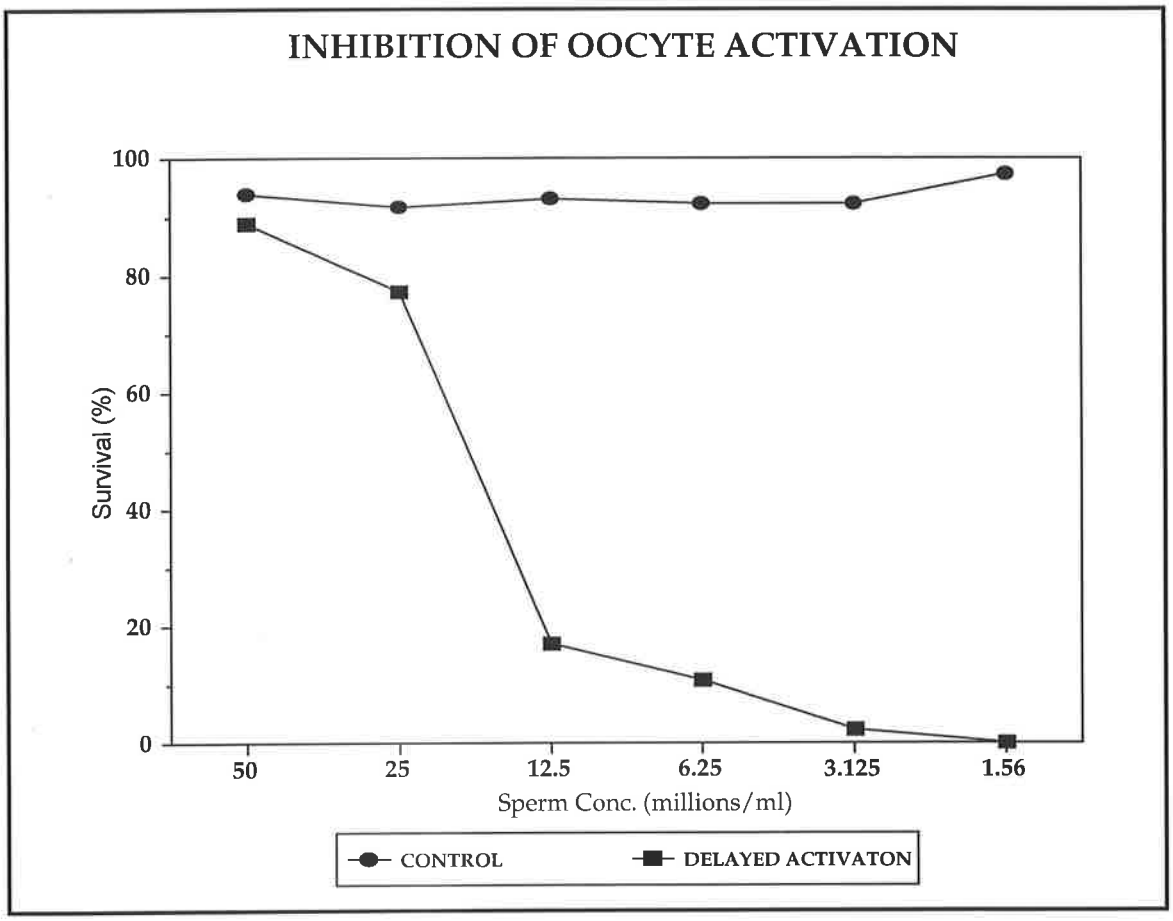


Figure 3.2 Inhibition of oocyte activation; effect of sperm concentration on subsequent fertilizability.

Survival at 24h of zebrafish oocytes (metaphase II) inseminated with serial dilutions of a sperm suspension; activation was effected immediately (control) or after a 30 min delay. This is a graphical representation of data presented in Table 3.4.

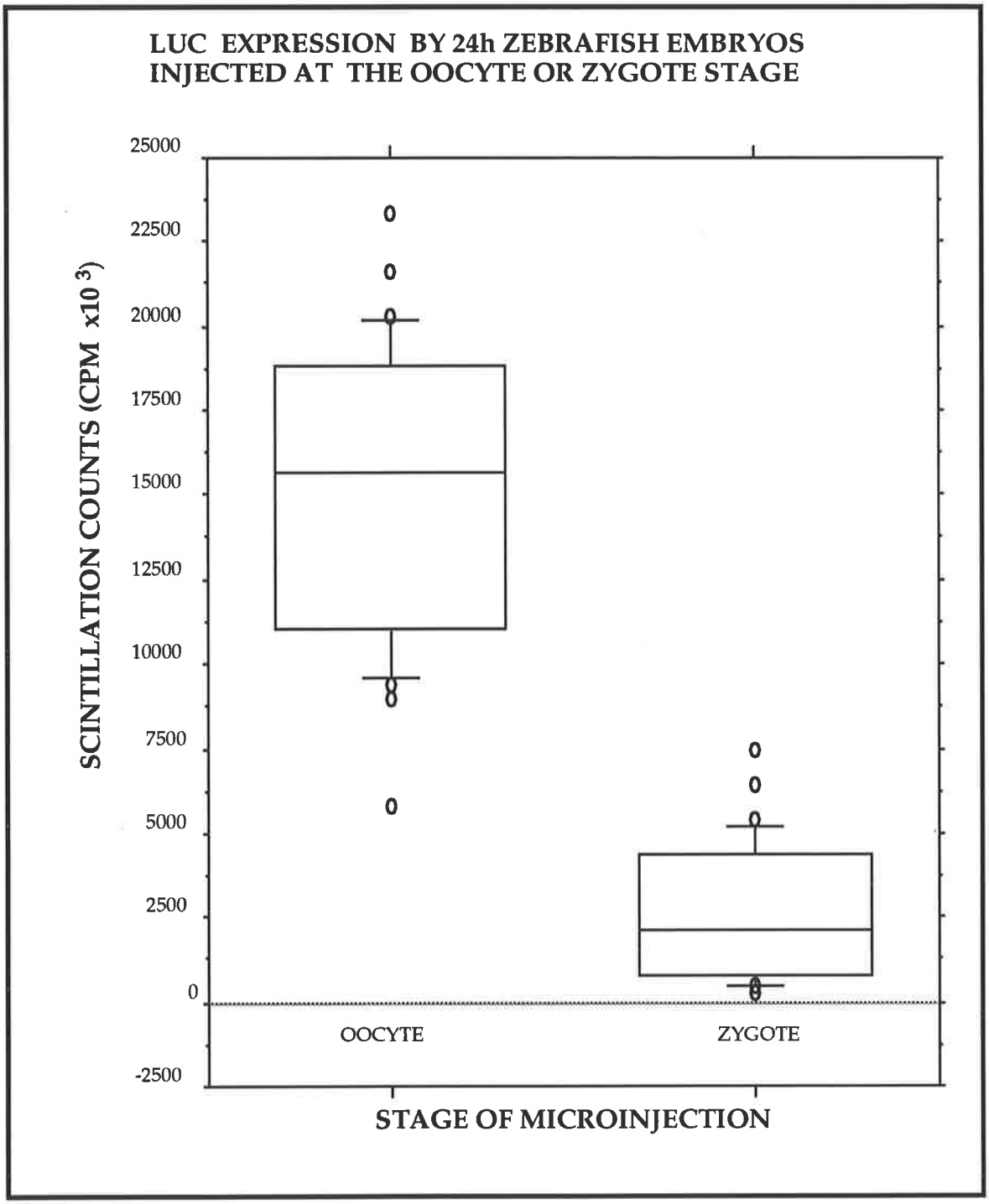
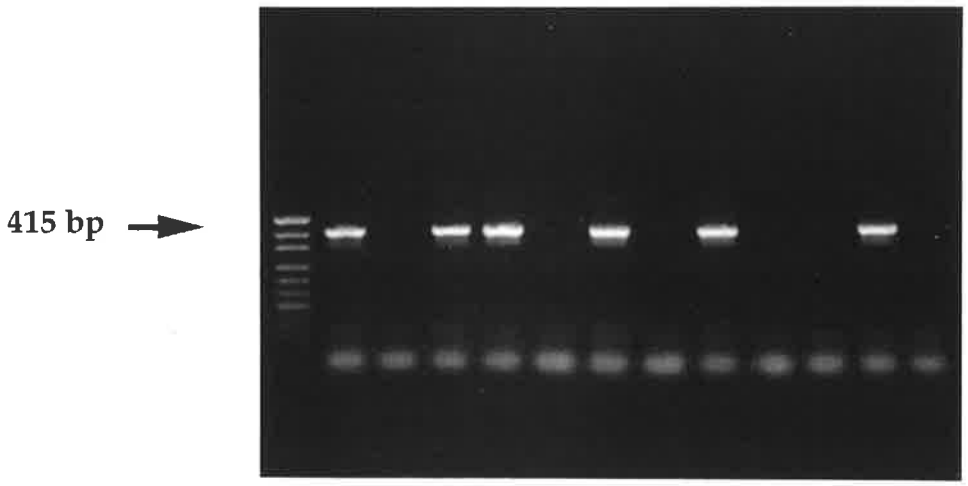


Figure 3.3 Expression of the pGL2 transgene by 24 h zebrafish embryos microinjected at either the oocyte or zygote stage of development.
Zebrafish eggs were microinjected with 600 pl of the pGL2 transgene at either the metaphase II oocyte or the zygote stage. Normal survivors were collected from each group and assessed for luciferase expression using a scintillation counter. The boxes represent the mean values with 95% confidence limits; outliers are also shown. Values for the two groups were significantly different (P,0.0001). The figure is a graphical representation of data sourced from Table 3.6.

Plate 3.1 Detection of the pGL2 transgene in fins of adult putative transgenic zebrafish

Genomic DNA from fins of adult (F0) zebrafish microinjected, with the linearized form of pGL2, at the metaphase II oocyte stage were analysed by PCR. 15 μl of the total reaction mixture after PCR was fractionated on a 2% agarose gel. The gel was stained with ethidium bromide and photographed under UV light. The arrow indicates the size of the expected amplified band (415 bp). Lane M: *Hpa*II digested pUC19 DNA size markers (DMW-P1; Bresatec, Australia). Lanes 1-9: Amplified genomic DNA from putative transgenic fish. Lane 10: DNA from an uninjected (control) fish. Lane P: Positive control sample containing DNA from a control fish mixed with 0.5 pg of linearized pGL2 plasmid, and Lane N: Reagent blank.

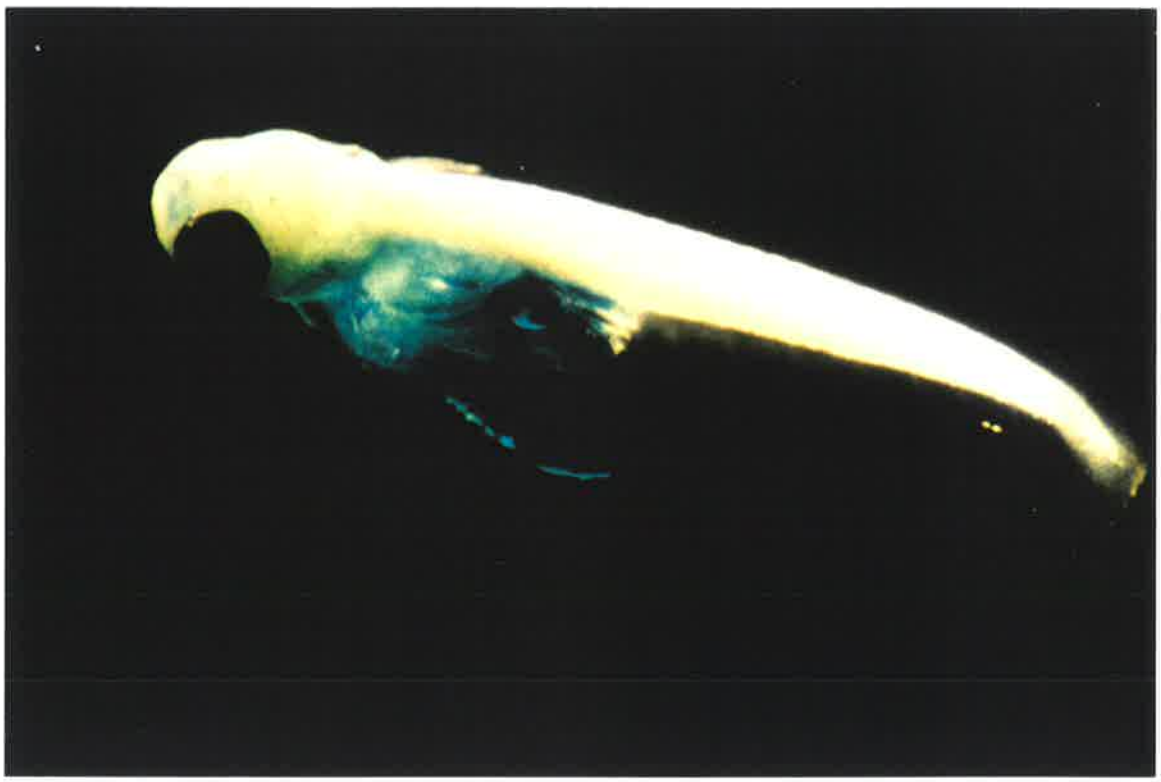
PCR Identification of pGL2 transgenic F0 zebrafish



Transgenic embryo	β-Gal expression (staining) seen in the otoliths and along the somites in a transgenic embryo, however the gut also stained deeply. NB: The staining of the somites that was clear in the originals, is not obvious in this photographic reproduction.

Control embryo	Control embryo showing deep staining only in the gut region presumably due to the bacteria present

Plate 3.2 Sperm mediated gene transfer in medaka *O. latipes*.
Embryos resulting from sperm incubated with the RSV β-Gal construct were analysed for expression of β-Galactosidase at Day 10 along with control embryos fertilised with sperm that were not exposed to the construct.



CHAPTER 4
PARTIAL GENOME MANIPULATION

4.1 INTRODUCTION

Gene targeting studies in mice, through homologous recombination (HR), have been facilitated by the availability of murine Embryonic Stem (ES) cells. ES cells (Evans and Kaufman, 1981; Martin, 1981) are totipotent cell lines derived from embryos, that can be cultured and genetically manipulated *in vitro* using conventional recombinant DNA procedures. They can then be re-introduced into mouse blastocysts, with the potential to contribute to all tissues in the resulting chimeric individual. When this includes the germline all the progeny derived from ES germ cells are of the desired altered genotype. These procedures are now routine for murine ES cell lines in many laboratories, but are still to be developed for livestock and other mammalian species of research and commercial interest (Seamark, 1994).

ES cell technology in fish is still in the 'embryonic' stage (Collodi *et al.*, 1992; Ozato *et al.*, 1995), and the primary aim of this study was to explore the possibility of creating chimeric embryos by transferring pluripotent embryonic cells from developing embryos into embryos at an equivalent stage. The success of chimera formation was to be determined by the alteration in pigmentation stemming from the use of cells from related fish with different pigmentation patterns.

Previously Ho and Kane (1990), had successfully transplanted cells between zebrafish embryos at the gastrula stage as a means of studying the behaviour of mutant cells. My aim was to attempt to perform blastomere transfers at an earlier stage of development when cells were still pluripotent (Kimmel and Warga, 1988).

4.2 CHIMERIC METHODS

4.2.1 Fish Species

The two species targeted for the study, *Danio rerio* (zebrafish, ZD) and *D. frankei* (leopard danio, LD), belong to the same genus. Adult *D. rerio* have a striped phenotype, and *D. frankei*, have pigmented spots in place of stripes, (Plate 4.9). A colour mutant form of *D. frankei*, called golden danio (GD), was also identified, which produced embryos that displayed a delay in the onset of pigmentation in their melanocytes, compared to wild type fish. In ZD and LD embryos raised at 26°C, pigmentation was found to appear at about 32h post fertilisation (pf); whereas, in GD embryos the onset of pigmentation only began after 50h. During subsequent development embryos from the three are indistinguishable, until the adult markings become evident by about 30 days of age (at 26°C). Preliminary experiments determined that matings between GD and either ZD or LD (regardless of cross) always resulted in progeny pigmented at ~32h of development indicating that a recessive mutation is responsible for the golden phenotype. Contribution of transplanted blastomeres of either ZD or LD origin, into GD recipient embryos, should therefore be able to be confirmed as early as 32h pf.

4.2.2 Supply of Embryos

Eggs and milt were obtained from ovulating females and spermiating males and artificially inseminated, following protocols outlined in general methods (section 2.3.1.2).

4.2.3 Micromanipulation for Production of Chimeras

4.2.3.1 Micromanipulation System

Manipulations were carried out using two three-dimension movement micromanipulators (NT-8; Narishige Scientific, Japan) attached to an inverted

microscope (Diaphot; Nikon, Japan) fitted with Differential Interference Contrast (DIC) optics. The objective turret was fitted with a LWD 20x, LWD 40x, and 10x DIC objectives and a 4x bright field objective. Microphotographs were either taken with a 35 mm automatic camera (Nikon) attached directly to a 35 mm bayonet fitting on the microscope, or with a camera attached to an Olympus SZ 60 stereo dissecting microscope using 50% of the total transmitted light. Photographs taken used 100 or 400 ASA colour slide and print film. To control meniscus movement in pipettes, two micrometer syringes (Gilmont Instruments, USA), 2.0 ml and 0.2 ml were attached to the blastomere-transfer pipette and holding pipette instrument holders respectively *via* thick walled plastic tubing (Masterflex Tubing 6409-16, Norton Performance Plastics, USA). The entire system was filled with silicon oil (200 fluid/20 centistokes, Dow Corning, USA).

4.2.3.2 Manipulation Pipettes

Blastomere Transfer Pipettes were manufactured from thin walled glass capillary tubing (GC-100T-15), directly from packaging (Clark Electromedical, UK). Capillary tubing of 150 mm in length was pulled on a pipette puller (P-77B; Brown-Flaming, USA) to produce a pipette with gradually tapering tips, which was cut on a microforge (Defonbrune, France) to an OD of 55 to 60 μm. The cut end was ground on a capillary grinder (Bachofer Laboratoriumsgerate, Germany) to produce a 45° bevel. The ground surface was heat polished followed by spiking of the distal tip of the bevel on the microforge. The holding pipettes used were similar to the ones used for microinjection (section 3.2.1.2).

Using the microflame or microforge all pipettes were bent twice 100° twice at 6 mm and 14 mm intervals from the tip. They were then stored in glass jars until required.

4.2.4 Methodology of Micromanipulation

Embryos at the mid blastula stage embryos (3-4h pf at 26°C) were used both as donors as well as recipients, for manipulation. Donor embryos, at the appropriate stage, were placed in the lid of a 36 mm plastic cell-culture dish (manipulation chamber) filled with Fish Ringer Solution, on the stage of the inverted microscope. The embryos were immobilised with the holding pipette, by applying suction with the Gilson syringe, with the animal pole facing the transfer pipette, which was then inserted through the chorion until it just penetrated the outer layer of cells. By applying suction on the transfer pipette blastomeres were gently drawn up, with care being exerted to avoid damaging the yolk sac. The donor embryo was released and exchanged for a recipient embryo. The donor blastomeres were subsequently injected into the recipient embryo and expelled beneath the outermost layer of cells, near the yolk sac (Plate 4.1, 4.2).

4.2.5 Manipulation media

Embryos were manipulated in Fish Ringer Solution (Ginsberg, 1963) (for the composition see section 2.1.2). Post manipulation, they were maintained until hatching (approximately 72h at 26°C) in 50% Fish Ringers (diluted 1:1 with Milli-Q water), after which they were transferred to tank water.

4.3 STATISTICAL ANALYSIS

Group data was analysed by ANOVA, subsequent to which individual values were compared using the Chi-Square test.

4.4 EXPERIMENTAL

4.4.1 Detection of Inter-Specific Chimeras of the Genus *Danio*, During Early Development

Aim 1: To determine whether blastomeres, isolated from developing embryos at the mid-blastula stage (3-4h pf), could be successfully transplanted to recipient embryos of a related species, at a comparable developmental stage, and contribute to the resulting manipulated (chimeric) embryos.

Aim 2: To examine whether pigmentation could serve as a useful means to identify chimeras early in development.

4.4.1.1 Outline

For this study zebra (ZD) and golden danio (GD) embryos were used as donor and recipient embryos, respectively. 20-50 ZD blastomeres from mid blastula (donor embryos) were injected into each GD (recipient) embryo. Unmanipulated control embryos belonging to both donor and recipient groups were cultured to provide an indication of egg quality and pigmentation. Donor embryos from which blastomeres had been removed and some embryos whose chorion was mechanically damaged, without disturbing the blastodermal cells were maintained as manipulated and chorion damaged controls, respectively to determine the suitability of the manipulation media.

Embryos belonging to all the groups were scrutinised under a camera mounted, stereo dissecting microscope (Olympus SZ 60), and the number of surviving embryos at 24h, was recorded, making a note of the incidence of deformity.

From the onset of pigmentation in controls belonging to the donor (ZD) group , (approximately 32h at 26°C) embryos belonging to all the groups were

observed microscopically and photographic records were maintained. This study was conducted as a series of three replicated experiments.

4.4.1.2 Results

Survival of chimeric manipulated embryos at 24h was high, but it was still significantly lower ($P<0.05$) than unmanipulated controls (77 and 94% respectively). The blastomere stage proved suitable for transplantation experiments, as the transplanted cells contributed at a high frequency (88%), to the resultant chimeric embryo, (Table 4.1). Lin *et al.*, (1992), also noted a high frequency of phenotypic chimeras (85%), at the equivalent stage, in similar experiments, in which they transplanted cells from pigmented to albino embryos of the same species (intra-specific). The experiments reported in this series of studies demonstrated that cells derived from blastula stage embryos of one species of the genus *Danio* are capable of surviving and proliferating in embryos of a related species, thus overcoming species barriers.

Pigmentation proved to be a convenient early marker of transplantation success (Plates 4.3, 4.4), with pigment expression of the transplanted blastomeres being uninfluenced by the surrounding cells belonging to a host species.

Pigmentation was first detectable in the eyes of 36h chimeric fish, with the pattern varying on either side of the same fish, this lateral variation was also seen for general body pigmentation in chimeras through to 55h of development. By contrast, control ZD embryos, were evenly pigmented on both sides, at the same stages and GD embryos displayed a lack of pigmentation (Plates 4.3 - 4.8).

4.4.2 Contribution of Transplanted Blastomeres in Adult Chimeras

Aim: To discern whether transplanted blastomeres derived from a related species, can survive during development and contribute to the resulting adult chimeras.

4.4.2.1 Outline

50-100 cells from mid-blastula stage ZD (pigmented) donor embryos were injected within the blastoderm of GD recipient embryos. Manipulated as well as unmanipulated control embryos were included as detailed in the previous series of experiments.

Embryo survival at 24h was recorded and the occurrence of deformity was noted. Manipulated embryos together with the appropriate controls were cleaned daily and raised to maturity, using the larval rearing techniques described in general methods (section 2.4). Five replicated experiments were conducted.

4.4.2.2 Results

For this series of experiments the number of donor cells transplanted was greater than the number transferred in the previous series (50-100 and 20-50, respectively), and this was reflected in a lowered survival rate for the chimeric group, however the values were not significantly different (Tab 4.4).

Due to a devastating protozoan infection, following a major month-long power failure in the Medical School, which affected the survival of larvae, only 14% (5/36) of the chimeric larvae survived to maturity (3 months). Both manipulated and unmanipulated control groups were equally affected with survival rates of 15 and 23% (5/43 & 14/62), respectively (Table 4.2). Even the few surviving fish died prior to mating, therefore the contribution of the donor blastomeres to the germline of the resulting chimeras could not be assessed.

Surprisingly, none of the 14 chimeras, surviving beyond day 30, the stage when adult ZD (donor) pigmentation was first apparent (Table 4.2), displayed a pigmented phenotype. Lin *et al.*, (1992), noted germline chimeras that failed to display a chimeric phenotype, however their study produced 33% phenotypic adult chimeras. Thus although the number that survived to the adult stage in this study was small, it was sufficient to indicate a lack of contribution of donor cells to the adult chimeras, possibly due to incompatibility of the donor and recipient embryos.

An important observation made while performing the manipulations, was that the GD (recipient) embryos consistently cleaved at a more rapid rate than the ZD donor embryos, with the result that all the recipient blastomeres were noticeably smaller than the donor cells, despite both batches of embryos being fertilised simultaneously. This raised the possibility of the transplanted cells being *'out-competed'* by the more rapidly proliferating blastomeres of the recipient embryo.

4.4.3 Efficient Production of Inter-specific Chimeras using Cleavage Advanced Embryos as Donors

Aim: To investigate whether comparative cleavage rates of donor and recipient embryos affect the contribution of transplanted blastomeres in resulting adult chimeras.

4.4.3.1 Outline

For this series of experiments ZD embryos were used as recipients and leopard danio (LD) embryos, which had a comparatively advanced cleavage rate (approximately 1 cleavage division faster in every 5), as the source of donor blastomeres. Eggs fertilised simultaneously were used for manipulation. 50-100 LD blastomeres were injected into recipient (ZD) embryos as described

previously. Manipulated and unmanipulated controls were maintained as controls of manipulation and egg quality respectively.

Embryo survival and the incidence of deformity at 24h was recorded for each group. All the surviving embryos from the chimeric group, and batches of embryos indicative of the control groups were reared to maturity.

By Day 30 (26°C), when adult pigment patterns (markings) became visible, chimeras and controls were visually examined, and the presence of stripes and/or spots was noted.

Adult chimeras were to be mated with wild type ZD to determine the contribution of the transplanted donor cells to the germline of chimeras; this study consisted of a series of four replicated observations.

4.4.3.2 Results

By using cleavage advanced embryos as the source of donor blastomeres, phenotypic 'inter-specific' chimeras, identified by adult markings at Day 30 (Plate 4.9-4.12), were produced at a high frequency of 40% (6/15), with 78% (14/18) of chimeric larvae surviving to maturity (Table 4.3) which was substantially higher than the that seen in the previous experiment (Table 4.2). Overall survival of the manipulated embryos in the chimeric group to maturity was 18% (14/79; Table 4.3). Pigmentation patterns of adult phenotypic chimeras also differed on both sides of the same fish as was earlier observed for chimeric embryos (Experiment 4.4.1), and the degree of chimerism varied greatly (4.10-4.12). Extensive donor contribution to chimeras often resulted in a degree of deformity (Plate 4.12).

4.4.4 Survival and Subsequent Development of Manipulated Chimeric embryos

4.4.4.1 Outline

Data for chimeric, manipulated and unmanipulated, as well as chorion damaged controls, from the preceding experiments was pooled and analysed. The rates of survival and the incidence of deformity observed in the different groups was compared.

4.4.4.2 Results

A comparison of the data accumulated from the three previous experiments with regard to survival and incidence of deformity, indicated that the overall survival, of embryos that received blastomeres varied from 65-80% and was not significantly different between experiments and the percentage of normal survivors were also similar in the groups (Table 4.4).

The increase in number of cells transferred from 20-50 in experiment 1 to 50-100 in the next two experiments was reflected by a significant ($P<0.05$) increase in the incidence of deformity observed among chimeras produced in the different experimental batches (Table 4.4).

Culturing chorion damaged embryos, in half strength (50%) Fish Ringers Solution, did not affect the survival of embryos significantly, compared with chorion-intact controls maintained in tank water (91 and 94% respectively), indicating it's suitability for these studies. The overall survival of manipulated embryos belong to both chimeric and control groups (73 and 72% respectively) was not significantly different, however they displayed a significantly lower ($P<0.001$) survival when compared to either chorion damaged or unmanipulated controls (Table 4.5).

The fraction of deformed embryos resulting from these studies, were consistently and significantly higher ($P<0.001$) for the chimeric group than the manipulated control group (23 and 9% respectively) (Table 4.5).

4.5 DISCUSSION

Successful transplantation of cells between zebrafish embryos has previously been achieved by Ho and Kane (1990), in a study aimed at comparing the movement and fate of wild type and mutant cells. The aim of this study was to adapt similar approaches for the purposes of gene manipulation studies, including transgenesis and gene targeting.

When this study was commenced there was little published in this area, therefore all the methodology had to be developed.

Pigmentation proved to be an extremely useful marker of the contribution of donor blastomeres to the chimera, providing an early indicator of the success of the manipulations and a measure of the degree of chimerism in the adult. The demonstration that blastomeres can be transplanted between related species, and survive to contribute to the resulting chimeric adults is encouraging for intended application of the technology to species of commercial interest.

Phenotypic adult *inter-specific* chimeras were produced at a frequency of 40%, which is comparable to the rate of 33% achieved by Lin *et al.*, (1992), for *intra-specific* chimeras of *Danio rerio*. Overall, 50% (119/238) of chimeric embryos in the present study survived the manipulation and developed into essentially normal larvae compared with 32% (135/418) survival reported by Lin *et al.*, (1992). This improvement in survival may have been due to the different manipulation approach used in the present study.

A number of significant variables were identified which could influence the end result of similar trials. These include the number of cells transferred, and the relative cleavage rate of donor and recipient embryonic cells. Increasing the number of cells transferred increased the ratio of deformity seen in resultant chimeric embryos. Notably, differing cleavage rates between donor and recipient embryos markedly influenced the fraction of donor cells that persisted into adulthood.

Fish Ringer solution proved to be a suitable medium in which to perform blastomere transplantation, however preliminary studies showed that when it was used for the culture of chorion damaged embryos to hatching, all the embryos developed abnormalities. Survival equivalent to unmanipulated controls, was achieved when chorion damaged embryos were cultured in a 50% solution of Fish Ringer's (Table 4.5).

The use of embryos at the blastula stage of development proved well suited to the purpose as the cells had not lost totipotency, and the even size of blastomeres at this stage (30-40 μm) simplified manipulations.

There was insufficient time to fully evaluation the contribution of donor cells (LD) to the germline of the chimeras at the time of writing this thesis, however most of the chimeras produced appear normal and healthy, and viable hybrids of the two species used for this series of studies, have previously been produced by simple artificial insemination (Kavumpurath and Pandian, 1992a), indicating the likelihood of the chimeras being sexually viable.

In vitro techniques allowing the establishment and maintenance of piscine ES cell lines still need refinement to realise the full potential of genetic manipulation through the ES cell approach (Ozato *et al.*, 1995). In the interim short term culture of totipotent blastomeres may be sufficient to allow genetic

manipulation *in vitro*. Also due to the high rates of transgenesis achieved by methods reported earlier in this thesis (see Chapter 3), it may be practical to attempt gene targeting and insertional mutagenesis, *in vivo* by injecting embryos directly and studies to determine the practicality of such an approach have been initiated in this laboratory (Cuong Do, per comm).

In conclusion this series of studies, has shown the possibility of creating chimeric fish, by mixing embryonic cells. Thus opening the way for genetic manipulations of the fish genome using a wide range of recombinant DNA techniques.

Table 4.1 The survival rates of embryos and frequency of pigmented chimeras formed following blastomere transfer.

Blastomeres from pigmented Zebra Danio donor embryos at the blastula stage were injected into unpigmented Golden Danio embryos at a comparable stage. The presence of pigmentation in embryos was determined at 36h by microscopic examination. For all experimental details see text..

Treatment Group	Eggs used n	Survival at 24h (%)	Pigmented embryos at 36h	
			n	%
EXPERIMENTAL	52	**77[a]**	35/40	**88***
CONTROLS				
RECIPIENT (GD)	51	**94[b]**	0/48	-
DONOR (ZD)	57	**93[b]**	53/53	**100**

Values within columns with different superscripts differ significantly **(P<0.05)**

* The distribution of pigment was not even in the embryos belonging to this group (See Plate 4.3-4.4).

Table 4.2 Survival to maturity of inter-specific chimeric embryos.
Comparison of survival to maturity of chimeric, and manipulated and unmanipulated control embryos.

Treatment Group	Embryos used	Survival at 24h	Larvae reared	Survival of reared larvae		
				Day 30# (Adult markings)	Day 60 - 90# (Sexual Maturity)	
	n	%	n	n	n	%
CHIMERAS	107	65	36	14	5	**14**
CONTROLS						
MANIPULATED	121	65	34*	7	5	**15**
UNMANIPULATED	161	94	62*	25	14	**23**

* Only a few larvae representative of the control groups were taken for rearing.
# High mortality was seen for the reared larvae in all groups, due to a severe protozoan infection.

Table 4.3 The production of chimeras using relatively more advanced embryos as the source of donor blastomeres. 50-100 blastomeres from leopard danio (LD) embryos that were cleaving at a faster rate than zebrafish (ZD) embryos were injected into ZD recipient embryos to produce chimeras. Phenotypic chimeras were visually identified when the larvae developed adult markings (~ Day 30).

TREATMENT GROUP	Embryos used	Survival at 24h	Larvae reared	Survival of Larvae at different stages of development		
				Day 30 (Adult markings)	Day 60 - 90 (Sexual Maturity)	
	n	%	n	n	n	%
CHIMERAS	79	80	18	15	14	78
CONTROLS						
MANIPULATED	86	65	12*	11	10	83
UNMANIPULATED	94	94	16*	15	14	88

* Only a few larvae from the control groups were taken for rearing.

Table 4.4 Effect of the donor cell-number on survival of chimeras.
Rates of survival and incidence of deformity observed in chimeras in the 3 series of experiments reported earlier in this study. Relevant statistical comparisons are made within the text.

Experiment Series no.	No. of cells transferred	Embryos n	Survival of embryos at 24h /(%)		
			total	normal	deformed
4.1	20-50	52	40 (77%)	32 (62%)	8 (15%)
4.2	50-100	107	70 (65%)	49 (46%)	21 (20%)
4.3	50-100	79	63 (80%)	38 (48%)	25 (32%)

Table 4.5 Comparison of overall survival and deformity observed in embryos from all the different treatment groups maintained for the study.

Values for the various control and experimental groups collected in the entire series of experiments were collated and presented.

TREATMENT GROUP	Embryos used n	Survival at 24h* n	Survival at 24h* %	Abnormality at 24h n	Abnormality at 24h %
CHIMERAS	238	173	**73[a]**	54	**23[c]**
CONTROL GROUPS					
MANIPULATED	278	201	**72[a]**	24	**9[d]**
CHORION DAMAGED	90	82	**91[b]**	0	-
UNMANIPULATED	363	340	**94[b]**	0	-

Values within columns with different superscripts differ significantly **(P<0.001)**.

* Overall survival including normal and abnormal survivors.

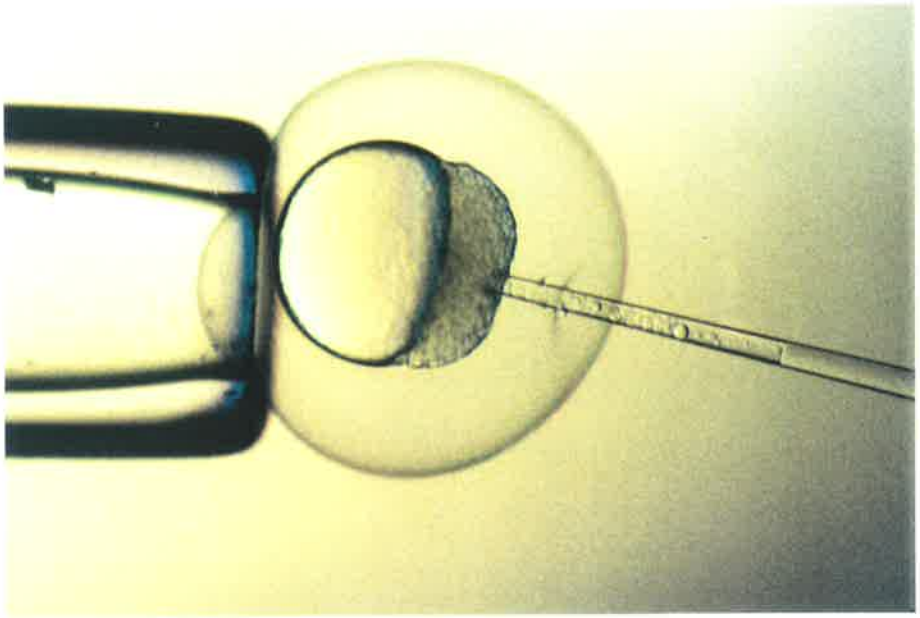
Removal of donor blastomeres

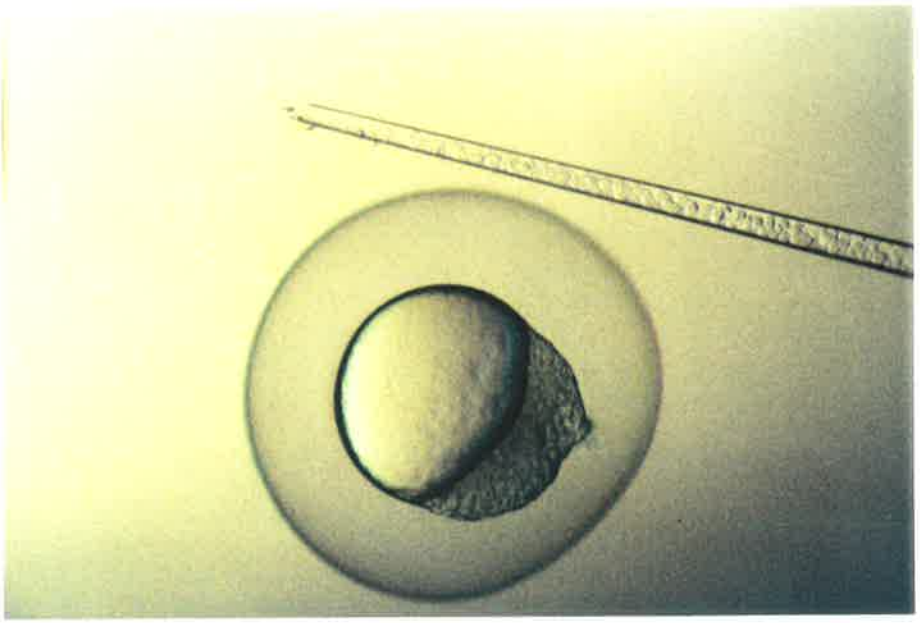
The embryo was immobilised with the holding pipette and the blastomere-transfer pipette was inserted through the chorion into the cell-mass near the yolk, and blastomeres were carefully drawn into the pipette with care taken not to damage the yolk...

Donor embryo after blastomere removal

After removal of blastomeres the transfer pipette with the cells was withdrawn through the chorion...

Plate 4.1 Removal of blastomeres from donor embryos.





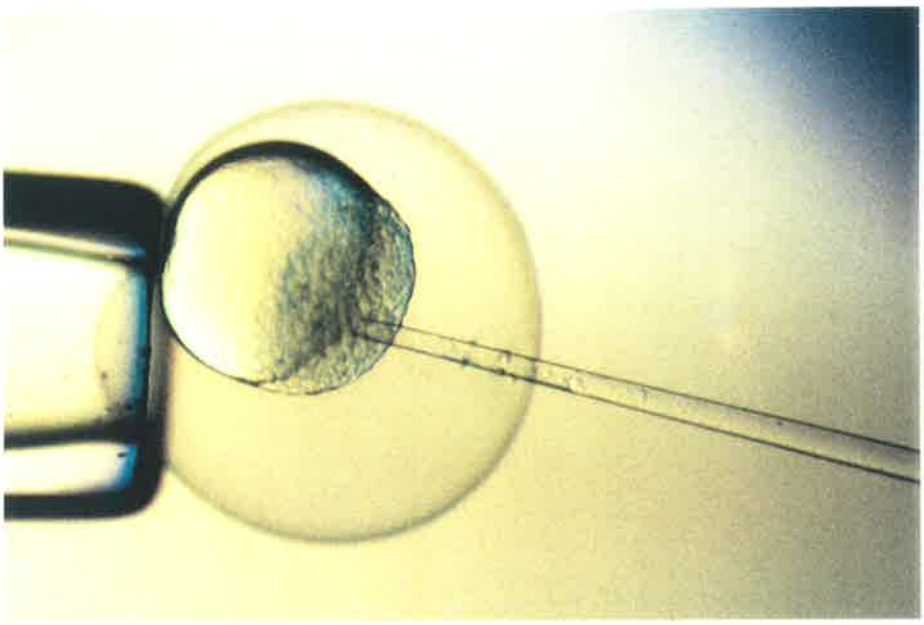
Injection of donor blastomeres

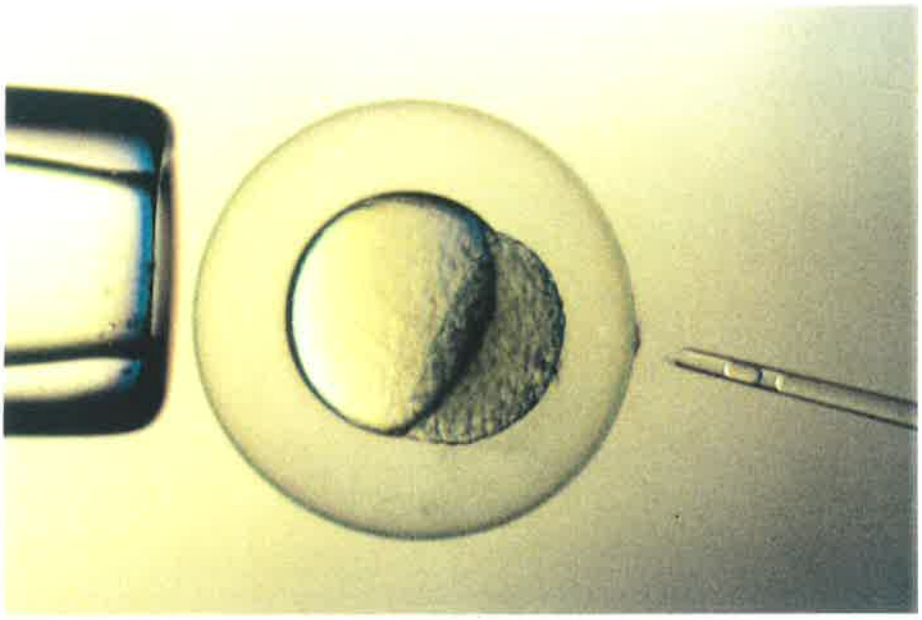
The recipient embryo was immobilised with the holding pipette and the blastomere-transfer pipette was inserted through the chorion into the cell-mass near the yolk, and donor blastomeres were injected with care taken not to damage the yolk...

Recipient embryo after blastomere transfer

After the injection of the donor blastomeres the transfer pipette was withdrawn carefully so as not to allow the injected cells to stream out of the recipient cell-mass when the pipette was removed.

Plate 4.2 Injection of donor blastomeres into recipient embryos.





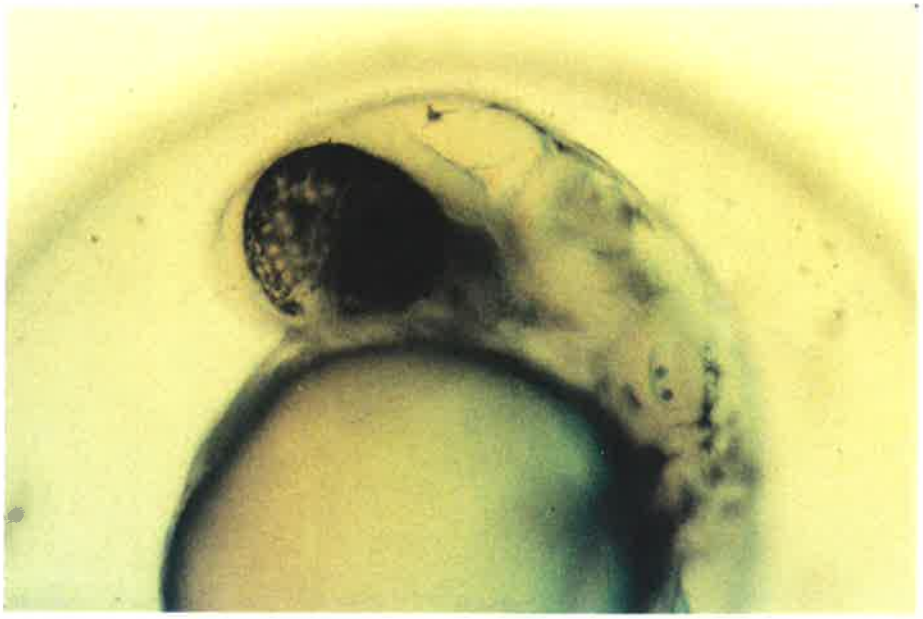
Control zebra danio (ZD) donor embryo

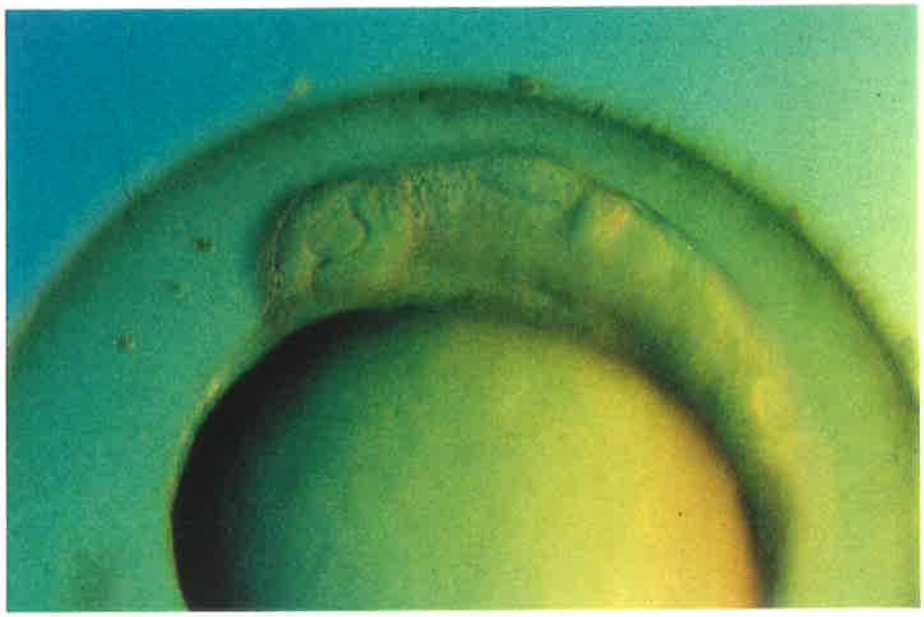
Typical ZD donor embryo with even pigmentation present in the eyes

Control golden danio (GD) recipient embryo

Typical GD embryo displaying a lack of pigmentation in the eyes

Plate 4.3 Chimeric manipulations: Control embryos at 36h of development





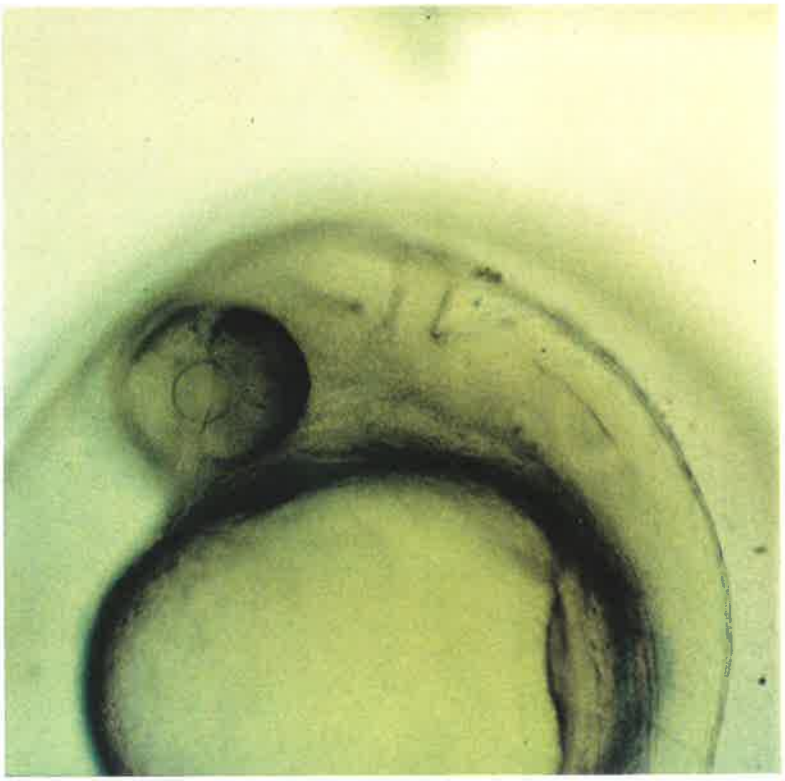
Chimeric embryo; left eye

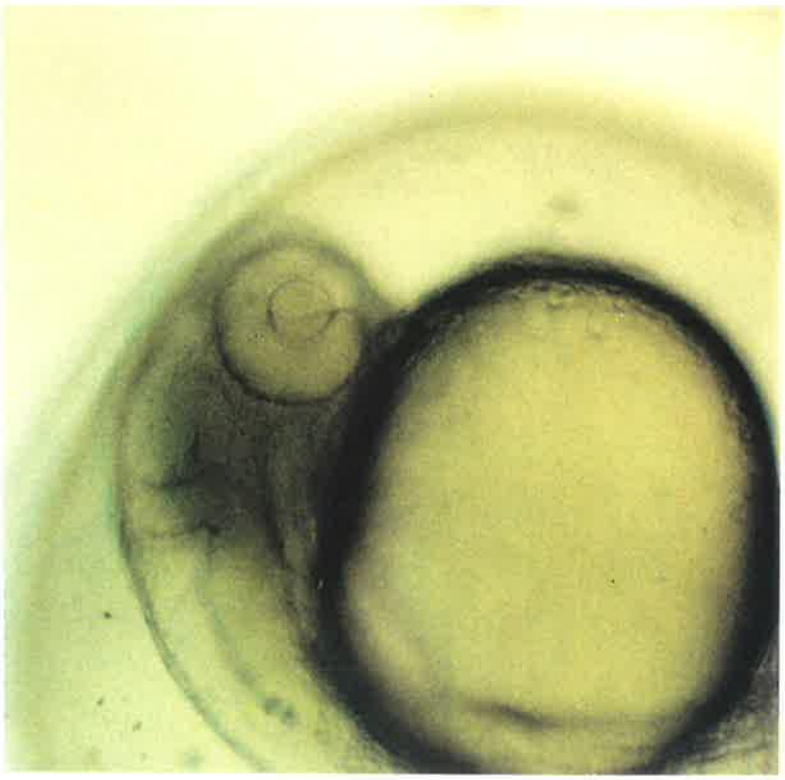
The left eye of a ZD-GD chimera with partial pigmentation apparent

Chimeric embryo; right eye

The right eye of the same chimera pictured above showing a lack of pigmentation

Plate 4.4 Chimeric manipulations: Chimeric embryo at 36h
Chimeras resulting from injection of zebrafish blastomeres into golden danio (GD) recipient embryos as a similar stage of development (blastula)





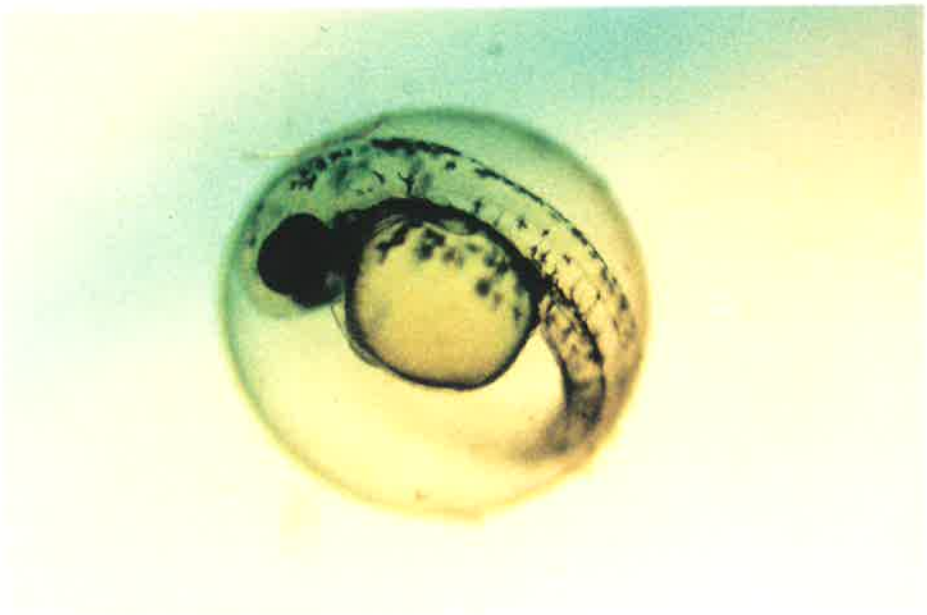
Control donor (ZD) embryo

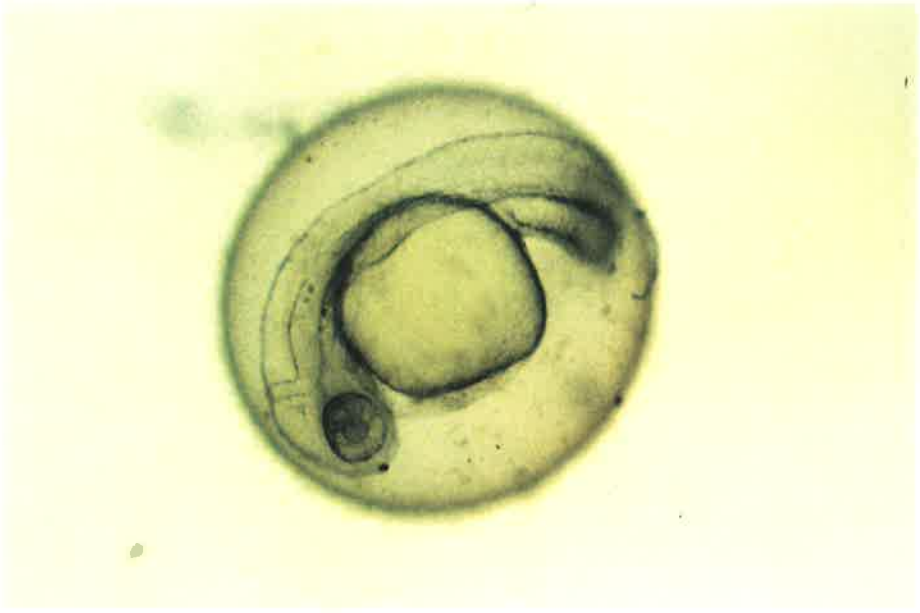
Typical ZD donor embryo with heavy and even pigmentation of the eyes and body

Control recipient (GD) embryo

Typical GD embryo with no pigmentation apparent on the body and eye pigment just appearing

Plate 4.5 Chimeric manipulations: Control embryos at 48h



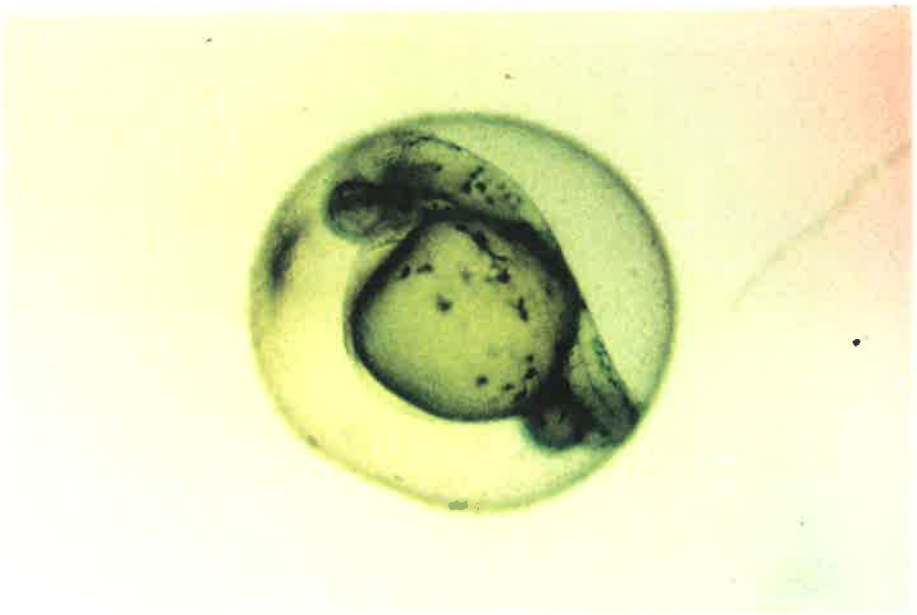


Chimera with an obvious contribution of donor (ZD) blastomeres. Note the uneven pigmentation in the eyes and scattered pigment on the body

The chimera on the left with pigmentation only noticeable in the eyes, contrasts with the one on the right that was obviously a phenotypic chimera. The pinwheel pattern of pigmentation noticed in the eye of this chimera was relatively common for the chimeric group

Another phenotypic chimera displaying contribution of donor blastomeres

Plate 4.6 Chimeric manipulations: Chimeric embryos at 48h



Control donor (ZD) embryo

Typical ZD donor embryo with heavy and even pigmentation of the eyes and body

Control recipient (GD) embryo

Typical GD embryo with no pigmentation apparent yet on the body and eye displaying a low level of pigmentation

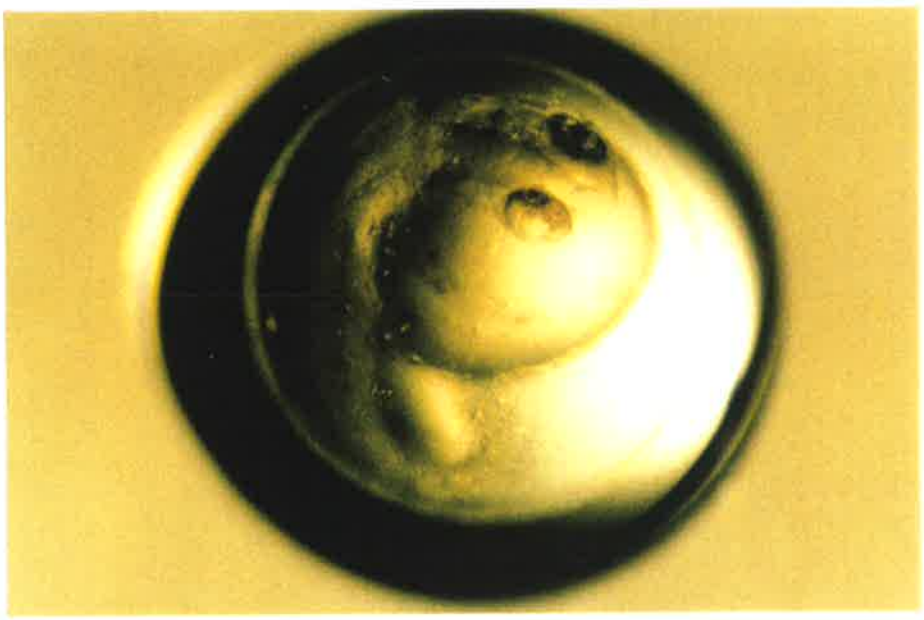
Plate 4.7 Chimeric manipulations: Control embryos at 55h

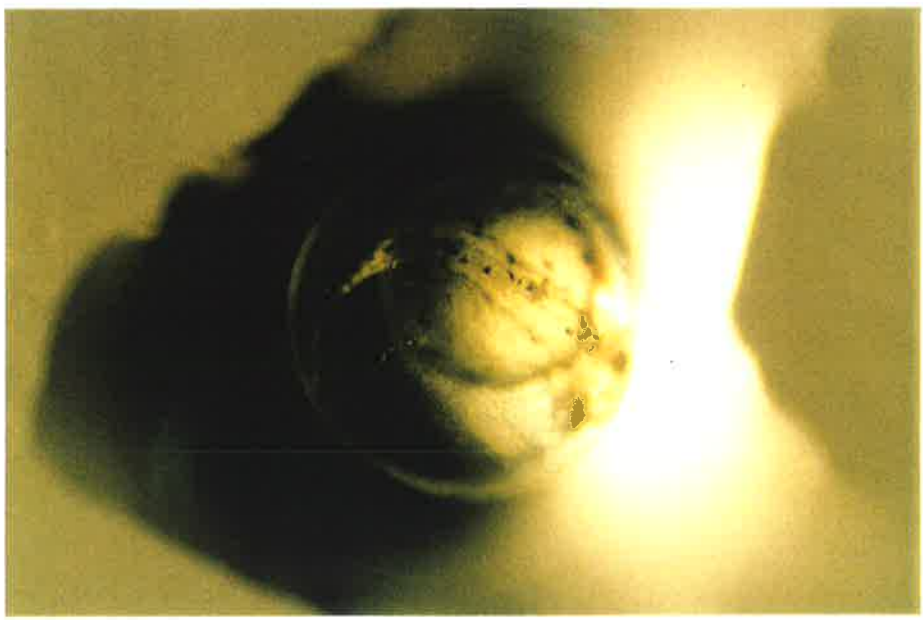
55h ZD-GD chimeric embryo showing a the lateral variation in pigment intensity in the eyes

A hatched chimera with a scattered pattern of pigmentation. The hole made in the chorion by the transfer pipette induced premature hatching in some manipulated embryos

A phenotypic chimera with increased pigmentation on the LHS of the body. The photograph presents a dorsal view of the chimera

Plate 4.8 Chimeric manipulations: Chimeric embryos at 55h





Control ZD recipient

Typical adult ZD displaying an adult phenotype of unbroken silver and blue stripes running from head to tail. ZD were used as a source of recipient embryos for these manipulations

Donor leopard danio (LD)

Typical LD displaying the adult phenotype of rows of metallic blue spots running down the lateral line. LD embryos were used as a source of donor blastomeres for this series

Plate 4.9 Chimeric manipulations: Control adult fish

Left hand side (LHS)

The pattern of stripes characteristic of the recipient (ZD) phenotype was broken in this male chimera

Right hand side (RHS)

The markings on this side varied from those on the LHS as was observed for chimeras at the embryonic stages

Plate 4.10 Chimeric manipulations: LD-ZD adult chimera 1 (male)
NB Part of the caudal fin of this fish was damaged during photography.

Left hand side (LHS)

Phenotypic chimeric female

Right hand side (RHS)

Bi-lateral variation apparent which was consistent for all phenotypic chimeras

Plate 4.11 Chimeric manipulations: LD-ZD adult chimera 2 (female)

Left hand side (LHS)

The increased contribution of donor (LD) blastomeres in this chimera is obvious. The low level of deformity seen for this fish appeared to be a reflection of the this increase

Right hand side (RHS)

Pigmentation patterns varied on both sides of the same fish

Plate 4.12 Chimeric manipulations: LD-ZD adult chimera 3 (male)

CHAPTER 5
INDUCTION OF TETRAPLOIDY IN THE ZEBRAFISH *DANIO RERIO*

5.1 INTRODUCTION

The enhanced growth and sterility of some triploid fish strains has attracted considerable commercial and research interest, and could be of great value as a means of controlling the reproduction of transgenic fish. The applications of triploidization have been well documented (Thorgaard & Gall, 1979; Thorgaard, 1983; Utter *et al.*, 1983; Don & Avtalion, 1986; Thorgaard, 1986; Arai & Wilkins, 1987; Kavumpurath & Pandian, 1990; Vardaraj & Pandian, 1990) but are presently limited by the deleterious effects of the treatments used to produce a high frequency of triploids, and the need to test each individual to verify successful manipulation.

In the fish species previously investigated triploidy has been usually achieved by polar body retention. In salmonids, it has proven possible, to produce triploids through a tetraploid-diploid mating and Chourrout *et al.*, (1986b), have achieved a high rate of success (> 93%), using this novel approach. The growth rates and viability of triploid rainbow trout produced in this way compared favourably to triploids produced by polar body retention (Myers *et al.*, 1986) but attempts to produce viable tetraploids in non-salmonid fish have not yet been successful (Myers 1986; Cassani *et al.*, 1990).

Studies reported in this thesis were undertaken to investigate the factors that affect the induction of tetraploidy in the zebrafish. In the absence of non-invasive ways of identifying tetraploid (4N) individuals, previous researchers have opted for an approach where the greatest tolerable shock treatment is used to maximise the rate of induction of tetraploidy and all the experimental embryos had to be raised to a size where a tissue sample can be obtained to

determine the ploidy status. Using this approach to induce tetraploidy in the grass carp Cassani *et al.*, (1990), noted a high percentage of 4N-2N mosaics among their treated animals, which they considered to have contributed to the lack of survival of 4N larvae. The pressure shock treatment used also posed technical problems and required the use of specialised equipment which limited the number of eggs that could be treated at a time, thus greatly reducing the potential benefit of this technology to the aquaculturist.

The aims of the present experiments were to-

1. Define parameters for temperature-induced production of tetraploid zebrafish,
2. Develop a non-invasive technique to identify 4N individuals based on embryo cleavage studies,
3. Identify tetraploid individuals early in development and optimise conditions, enabling maximum survival of ploidy manipulated individuals by minimising the treatment duration, and
4. Assess the survival and viability of tetraploid individuals.

5.2 PLOIDY MANIPULATION METHODS

5.2.1 Heat Shock treatment

Fertilised zebrafish embryos were obtained following AI procedures described earlier. Time 0 was established as the time when the gametes were activated with the addition of tank water. Embryos were maintained at 26°C in 36 mm disposable tissue-culture dishes on a warming tray until they were subjected to heat shock (HS).

Thick-walled (5 cm) polystyrene containers, of the kind that are used to transport reagents in dry ice, were observed to maintain the temperature of water stored in them for 20-30 min. The polystyrene container was half filled

with 4-5 litres of tap water at the predetermined (HS) temperature, and a 500 ml glass beaker (HS chamber) filled with 300 ml of tank water was floated in the container and allowed to reach the predetermined temperature. The temperature of the water in the beaker was monitored continuously with a digital temperature probe (Digitemp D2025; TFA, Germany).

Prior to the specified time of commencing the HS treatment the embryos were randomly selected for each treatment group and transferred from the culture dishes to a tea infuser, of the type available at department stores, which was placed in a beaker of tank water maintained at 26°C, on the warming tray. The beakers and the corresponding infuser containing the embryos were labelled appropriately. At the specified HS time post fertilisation (pf), the infuser was allowed to drain and placed in the HS chamber for the preset duration of shock, following which the infuser with the embryos was returned to the beakers on the warming tray. Treated embryos were recovered from the infusers and maintained at 26°C in culture dishes until they were analysed.

5.2.2 Analysis of ploidy

The ploidy status of zebrafish embryos was verified by karyotyping and counting metaphase chromosome spreads of 24-48h embryos prepared as described in general methods (section 2.5.4).

5.3 STATISTICAL ANALYSES

Group data was analysed using ANOVA. Individual values were subsequently analysed by the Chi-Square test.

5.4 EXPERIMENTAL PROCEDURES

5.4.1 Parameters for temperature induced tetraploidy in zebrafish

Aim: To identify and optimise parameters to induce tetraploidy in zebrafish, utilising Heat Shock treatment.

5.4.1.1 Outline

Fertilised eggs were obtained by AI and maintained at 26°C. Batches of eggs were subjected to a single heat shock (HS) of 41 ±0.5°C for a duration of 2 minutes, commencing at one min intervals. Six treatment groups with HS initiated from 9-14 min pf were evaluated. Control eggs were handled the same as the experimental except for being subjected to the HS treatment.

Embryos were examined microscopically at 24 and 48h pf, survivors were counted and the number with a normal morphology were recorded. The number of abnormal embryos was also noted. At least 40% of the surviving embryos from each experimental group were subjected to karyo-analysis at 48h pf and the numbers of diploid (2N), polyploid (Aneuploid, *ie* embryos with an intermediate chromosome number between 2N and 4N, and 2N-4N ploidy mosaic individuals were included in this group called polyploid for this study), and tetraploid (4N) embryos were recorded for each group. 26% of embryos from the group that were not subjected to HS were also karyo-typed as a control. This study was conducted as a series of three replicates.

5.4.1.2 Results

Survival of embryos to the pigmented eyed stage (48h pf) was highly variable in different treatment groups, ranging from 33-67% (ave 57%). Overall, survival of HS treated embryos was significantly ($P<0.001$) lower, when compared with untreated controls (57 and 90%, respectively). Also, 22% of the survivors from the treatment groups were morphologically abnormal at this

stage, where as none of the individuals in the control group developed abnormally (Table 5.1). The percentage of survivors that were normal was inversely proportional to the rate of induction of tetraploidy (Fig 5.1).

The rate of induction of tetraploidy varied greatly between groups, ranging from 0-38% (ave 17%), with the treatment group where shocks were initiated at 11 and 12 min pf consistently yielding ($P<0.05$) higher rates of tetraploids than any of the other treatment groups. Therefore these two groups, with tetraploid induction rates of 38 and 20% respectively (Table 5.2), were targeted for further studies. The incidence of polyploidy (includes aneuploid and 4N-2N mosaic embryos) was low (6%). The frequency of tetraploid induction in the HS treated groups was significantly greater ($P<0.001$) for larvae that were deformed, than for those with a normal morphology (56 and 7%, respectively; Table 5.3).

5.4.2 Cleavage rate as a gauge of incidence of tetraploidy

Introduction: Zebrafish eggs are known to exhibit synchronous cleavage until the 10th cleavage division (Laale, 1977), and we postulated that if HS treatment induced chromosome doubling by inhibiting the first cleavage division (cytokinesis), the tetraploid individuals could be identifiable on the basis of their cleavage rate being delayed by one division, when compared to untreated controls.

Aim: To determine whether the cleavage rate can be used as an accurate indicator of the ploidy status of a zebrafish embryo

5.4.2.1 Outline

Embryos that were subjected to HS treatment (as described in Experiment 5.4.1) were examined at the early cleavage stages (2-, 4- and 8-cell stage; 45, 60 and 75

min pf at 26°C, respectively) to determine whether the induction of tetraploidy resulted in delayed cleavage rate. The number with a normal, or delayed cleavage rate were recorded for both HS treated and control embryos. The embryos displaying a delay of one complete cleavage division (over two successive cleavage events) when compared with controls, were incubated separately and labelled as *delayed*. The morphology of all surviving embryos was recorded as in the previous experiment. Embryos from the control group, and the *delayed* experimental group as well as ones from the experimental group that did not display a delay in cleavage (*remaining*) were subjected to karyo-analysis. This experiment was performed as a series of five replicates.

5.4.2.2 Results

Overall, a total of 1325 zebrafish embryos were subjected to HS, of which 15% (202), were observed to trail untreated embryos by one complete cleavage division at both the 4-cell and 8-cell stage of embryo development (*delayed* group) (Table 5.4). The karyotype of 100% (68/68) of the analysed embryos from this group displayed a tetraploid chromosome set (4N = 100 chromosomes). In contrast only 4% (7/185) of the HS treated embryos that were not selected (*remaining*) contained a tetraploid chromosome number, with the majority (89%, 165/185) of the individuals from this group possessing a diploid set of chromosomes (2N = 50 chromosomes). All the embryos from the group not subjected to HS (controls) displayed a diploid karyotype.

The tetraploid state significantly ($P<0.001$) increases the rate of deformity as 58% (71/123) of the surviving embryos from the *delayed* group displayed an abnormal morphology at 48h of development, as opposed to the 25% (153/602) deformity seen in the *remaining* group which also experienced the same HS treatment (Table 5.4).

5.4.3 Maximising survival of tetraploids

Introduction: In previous studies relating to tetraploid induction, the difficulty of identifying tetraploid individuals early in their development, has lead to treatments being designed to yield maximal levels of chromosome doubling which may have compromised the viability of chromosome-set manipulated embryos. The ability to identify 4N embryos early in development (see experiment 5.4.2) facilitated the optimisation of the shock parameters, with a view to enhancing survival, as only the 4N embryos needed to be reared.

Aim: To maximise the survival of tetraploid zebrafish embryos by minimising the shock they are subjected to.

5.4.3.1 Outline

As results from experiment 5.4.1 demonstrated that HS (of 41 ±0.5°C) commencing at 11 and 12 min pf were most efficient at inducing tetraploidy, these stages were further examined. Preliminary experiments showed that a HS of 42 ±0.5°C for a 2 min duration resulted in 100% mortality whereas a HS of 40 ±0.5°C for the same duration (2 min) was ineffective in inducing chromosome doubling. Similarly subjecting embryos to a shock of a 1 min duration at 42 ±0.5°C was ineffective in inducing tetraploidy. Therefore, batches of embryos were heat shocked at 11 and 12 min pf, with a temperature shock of 41 ±0.5°C imposed for 1.5, 1.75 or 2 min. Embryos were randomly allocated to each experimental group including a group of untreated embryos that were maintained as controls. Those embryos that displayed a cleavage delay of one complete division were identified and reared separately. The number of embryos in each group, including controls, that developed normally to the pigmented eyed stage was recorded. Six replicated sets of observations were conducted.

5.4.3.2 Results

Varying the duration of HS significantly affected both the rates of induction of tetraploidy (16-49%, ave 30%; $P<0.001$) and also the normal survival of tetraploids at the pigmented eyed stage (48h) (3-14%, ave 10%; $P<0.05$). Shocks administered for a duration of 1.75 and 2.0 min, commencing at 11 and 12 min pf, respectively, were the most successful ($P<0.001$) in inducing tetraploidy (with induction rates of 42 and 49%, respectively). However the normal survival of 4N individual at 48h was maximal for both treatment groups when the HS was applied for a duration of 1.75 min (14 and 13%, respectively) (Table 5.5).

5.4.4 Survival and viability of Tetraploids

Aim: To rear 4N larvae, produced by methods developed in experiment 5.2.3., and to determine the survival and viability of tetraploid zebrafish.

5.4.4.1 Outline

Tetraploid embryos produced by methods employed in 5.4.3. were reared using standard procedures (see Chapter 2, section 1.6), survival and normality of larvae was recorded daily and comparisons made with control embryos reared under the same conditions.

5.4.4.2 Results

HS treatment of zebrafish embryos resulted in acceptable yields, of 31% (736/2387), of 4N individuals, overall. However a dramatic increase in mortality and deformity resulted with more than a 10 fold reduction in survival (736 at Day 0 down to 64 at Day 5) to the swim bladder inflation stage of development (Figure 5.2). The rates of early survival of, HS induced, tetraploid zebrafish were low, with deformities in tail development and heart formation contributing to the majority of deformed individuals (see Plate 5.1). A fraction,

3% (64/2387), of HS treated embryos developed normally see (Plate 5.1), underwent swim bladder inflation and commenced feeding, however their impaired viability became more pronounced with increasing age, with 92% (59/64) succumbing within the first 2 weeks of larval development. The few individuals that survived beyond this stage were noticeably subvital when compared with controls, being both shorter and thinner, they also displayed a more heavily pigmented phenotype when compared with controls. Only 1 individual survived to Day 48 (Figure 5.2). Control (untreated) embryos that were reared displayed normal viability and survived to maturity at acceptable rates of 80% (55/67).

5.5 DISCUSSION

Tetraploidy has been touted as a possible alternative route to producing triploids via a 4N- 2N mating (Refstie *et al.*, 1977; Chourrout, 1984). Most of the success with producing tetraploids has been achieved with salmonids utilising both HS (Thorgaard *et al.*, 1977; Chourrout, 1982; Chourrout, *et al.*, 1986b), and pressure shocks (Chourrout, 1984; Chourrout *et al.*, 1986b; Myers *et al.*, 1987; Foisil & Chourrout, 1992), and mortality of tetraploids in all the above studies has always been high. When these 4N individuals were mated with normal diploids, the resulting progeny consisted almost entirely of triploids (Myers & Hershberger, 1991).

In non-salmonids tetraploidy has been induced by a combination of pressure and temperature shock treatments, in *Oreochromis* species (Myers, 1986). The incidence of tetraploidy was low (5%), and survival to the eyed stage was highly variable, ranging from 9.9-67.5%. The 4N individuals were markedly sub-vital, and all succumbed within 7 days of hatching. Cassani *et al.*, (1990) also reported 100% mortality during development, of their 4N grass carp. They reported a high incidence of tetraploidy in both pressure and HS treatment

groups (25-100%). In the present study the survival of HS treated zebrafish embryos to the eyed stage was significantly lower ($P<0.001$) than controls (57 and 90% respectively), with surviving tetraploids being sub-vital and displaying increased deformity especially enlargement of the heart and peritoneal cavity, curvature of the tail and inhibition of swim bladder inflation.

In contrast to Chourrout's, (1986b), hypothesis, that HS affects cellular mechanisms that produce polyploids completely or inhibit the process altogether, Cassani *et al.*, (1990), observed 2N-4N mosaics at varying frequencies in most of their HS and pressure shock treatment groups, and they have attributed the sub-vitality of their tetraploids to problems of incomplete polyploid conversions, resulting in 2N-4N mosaics and aneuploid individuals. In the present study also, 2N-4N mosaics and aneuploids were identified, but at a relatively low frequency (6%), therefore they cannot be the only reason for the sub-viability of the tetraploids in this study.

Valenti, (1975), observed several tetraploids among blue tilapia eggs treated with a cold shock. Reddy *et al.*, (1990), working with two major Indian carps species, found both HS and cold shock to be effective in inducing tetraploidy in one species *Labeo rohita*, while for the other species *Catla catla*, only HS was successful. However preliminary work in this study (data not shown) found cold shock to be ineffective as a means of inducing tetraploidy in zebrafish.

There is no report in the literature of a non-invasive method of identifying tetraploids early in development. The demonstration that it is possible to identify tetraploid individuals at early cleavage stages as reported in this study, has great potential for adaption to a number of fish species. The availability of this approach was a key factor in optimisation of shock parameters, which resulted to a significant increase in the rates of induction and early survival of tetraploid zebrafish embryos.

Tetraploid zebrafish larvae were found to be sub-vital at all stages of development when compared with controls and despite minimising the HS parameters no survival beyond 48 days of development was observed.

The predominantly diploid chromosome number and normality of the unselected embryos in the experimental groups, suggests that the increased mortality of the selected 4N individuals was the result of the tetraploid state and not the HS treatment. However, there are several studies both in salmonids (Chourrout, 1982; Chourrout, 1984) and non-salmonids (Flajshans *et al.*, 1993) which show that pressure shock is more effective than HS treatments as a means of developing tetraploids and a specific effect of HS treatment as a factor in lack of survival of tetraploid zebrafish cannot be discounted.

One interesting observation was made on one batch of fish which showed low levels of tetraploid induction (data not shown). This batch had been maintained in a tank in which the pH was noted to be very high (8.2), and it was of interest to find that on reducing it to more normal levels (6.8-7.2), the rates of induction of tetraploidy increased to more usual values.

The early identification of tetraploids, by methods devised in this study opens up numerous possibilities, such as the production of 4N-2N chimaeras, using 4N embryos as a source of donor blastomeres, as an alternate route to production of triploids for this species.

Using information gathered in this study, the zebrafish embryo provides an excellent model for investigating the effects of chromosome doubling during early vertebrate development.

In conclusion, these studies have demonstrated the feasibility of using HS as a means of inducing chromosome doubling in the zebrafish. A significant

contribution has been made in devising a non-invasive method of identifying tetraploid individuals very early in development, which is applicable not only to fish but other vertebrates as well. This study clearly demonstrates that the severity of the shock treatments in these type of experiments, compromises the viability of chromosome-set manipulated embryos.

Table 5.1 Survival of embryos following heat shock (HS) treatment administered at varying times post fertilisation.

Zebrafish embryos produced by artificial insemination were randomly allotted to either one of the six treatment or the control group. Eggs were heat shocked at 41.5 ±0.5°C for a duration of 2 min. Batches of embryos from 9 to 14 min post fertilisation were exposed to heat shocks initiated at one minute intervals. Control embryos were handled identically except that they were not subjected to heat shocks. Embryos were maintained at 26°C and at 48h pf, embryos belonging to each group were examined microscopically and rates of survival and normality were determined.

Treatment group	Eggs Treated	Development of surviving embryos at 48h post fertilisation				
		Total Survival		Appearance*		Normal Survival
		n	%	N	A	%
9 min	60	31	52	26	5	43
10 min	57	19	33	16	3	28
11 min	142	78	55	52	26	37
12 min	176	110	63	81	29	46
13 min	93	57	61	46	11	50
14 min	75	50	67	47	3	63
Total	603	345	57[a]	268	77	13
Control	167	151	90[b]	151	-	90

Values within columns with different superscripts differ significantly **(P<0.001)**

* Based on visual inspection, N = normal embryos : A = abnormal embryos

Table 5.2 Induction of tetraploidy in zebrafish.
48h old embryos from each of the experimental and the control group were subjected to karyo-analysis. Chromosome spreads were prepared as described in general methods (section 2.7), and the ploidy status was determined based on chromosomal counts of at least 5 metaphase spreads representative of each larvae.

Treatment	Eggs Treated	Survival at 48h	Analysed	Ploidy Status		
				Diploid	Polyploid	Tetraploid
Group	n	%	n	%	%	%
9 min	60	52	22	73	18	9[b]
10 min	57	33	10	90	10	-
11 min	142	55	37	57	5	38[a]
12 min	176	63	45	80	-	20[a]
13 min	93	61	23	83	9	9[b]
14 min	75	67	23	96	4	-
TOTAL	603	57	160	77	6	17
CONTROL	167	90	40	100	-	-

Values within columns with different superscripts are significantly different **(P<0.05)**

Table 5.3 Frequency of tetraploid zebrafish in normal and abnormal embryos following HS treatments.
Surviving embryos from the experimental and control groups following HS initiated at varying times pf were allocated to either normal or abnormal groups based on their morphology. The ploidy status of the analysed embryos was determined by karyotyping.

Treatment group	Number Treated	Surviving embryos at 48h post fertilisation									
		Normal morphology					Abnormal morphology				
		n	No. Analysed	D* %	P* %	T* %	n	No. Analysed	D* %	P* %	T* %
9 min	60	26	17	94	6	-	5	5	-	60	40
10 min	57	16	8	100	-	-	3	2	50	50	-
11 min	142	52	27	78	-	22	26	10	-	20	80
12 min	176	81	33	91	-	9	29	12	50	-	50
13 min	93	46	20	95	5	-	11	3	-	33	67
14 min	75	47	23	96	4	-	3	0	-	-	-
TOTAL	603	268	128	91	2	**7[a]**	77	32	22	22	**56[b]**
Control	167	151	40	100	-	-	0	-	-	-	-

* Ploidy status of embryos based on karyo-analysis, D = diploid; P = polyploid; T = tetraploid.
Values within the table with different superscripts are significantly different **(P<0.001).**

Table 5.4 Cleavage rate as an indicator of tetraploid status.
Experimental and control embryos were maintained at 26°C. At 60 and 75 min pf the embryos belonging to both groups were examined microscopically. Embryos that displayed a delay in cleavage rate of one division over two consecutive cleavage events compared with controls were isolated, maintained separately and labelled as Delayed, the remaining unselected embryos in the HS treatment group were titled appropriately. The ploidy status of 48 h old embryos was determined by karyo-analysis. Relevant statistical comparisons are made within the text.

Treatment Group	Treated n		Survived 48h# n/(%)	N	A	Analysed n	Karyo-Analysis* Normal	Abnormal
HEAT SHOCK	1325	DELAYED	202 (15)	52	71	68	24^{T}	44^{T}
		REMAINING	1123 (85)	449	153	185	149^{D} 2^{P} 2^{T}	16^{D} 11^{P} 5^{T}
CONTROL	341			311	-	48	48^{D}	-

* Ploidy status based on karyotyping - ^{D} = diploid; ^{P} = polyploid; ^{T} = tetraploid

\# morphology based on microscopic examination - N = normal; A = abnormal

Table 5.5 Effect of duration of HS on induction and normal survival of tetraploids.
Batches of embryos were subjected to temperature shock with heat shock commencing at either 11 or 12 min pf. Heat shock of 1.5, 1.75 and 2.0 min duration were examined. the number of cleavage delayed (tetraploid) embryos and the normality of tetraploid embryos was noted for each experimental group.

Time of Initiation of HS (pf)	Duration of HS (mins)	Eggs Treated n	Tetraploids (Cleavage Delayed) n	%	Normal Tetraploid Embryos at 48h n	%
	1.5	433	93	**22^{b}**	36	**8^{y}**
11 min	1.75	519	218	**42^{a}**	71	**14^{x}**
	2.0	240	48	**20^{b}**	6	**3^{y}**
	1.5	313	51	**16^{b}**	17	**5^{y}**
12 min	1.75	296	79	**27^{b}**	39	**13^{x}**
	2.0	258	127	**49^{a}**	30	**12^{x}**

Values within columns with different superscripts are significantly different, **$^{a-b}$ = (P<0.001); $^{x-y}$ = (P<0.05)**

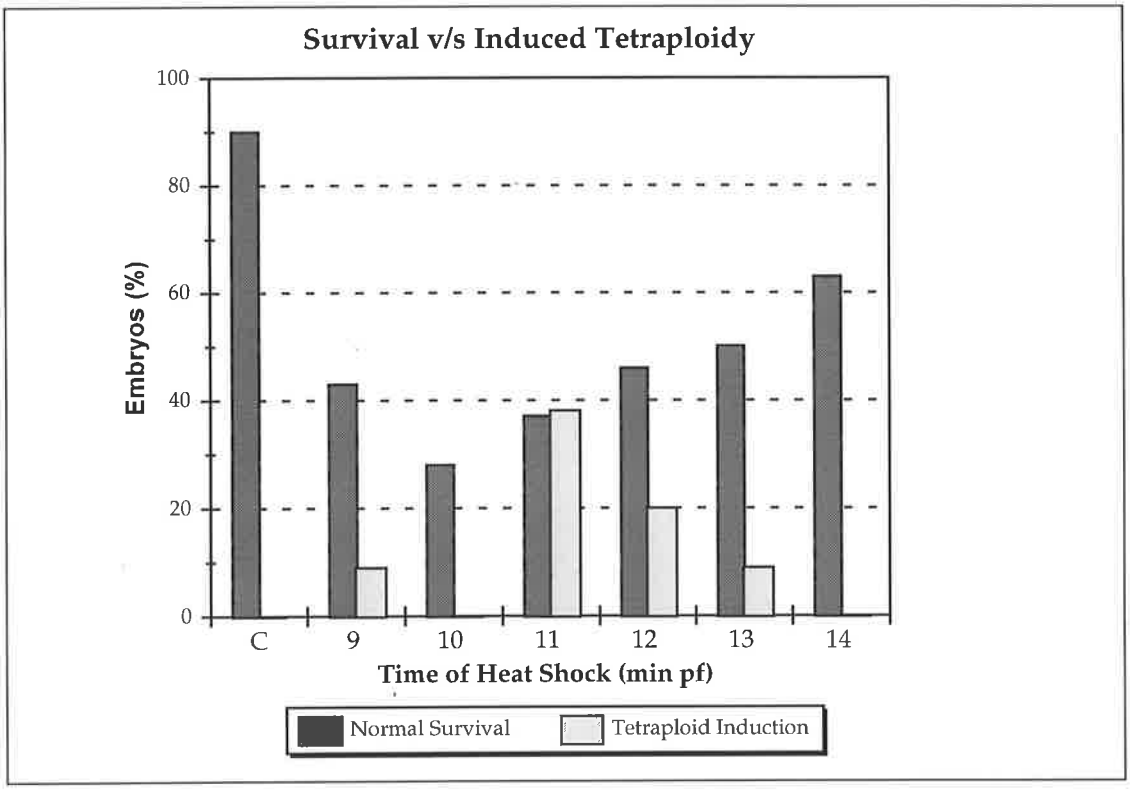


Figure 5.1 Normal survival is inversely proportional to tetraploid induction. Comparisons were made between the rate of normal survival (Table 5.1) and the rate of induced tetraploidy (Table 5.2) for zebrafish embryos following HS at varying times post fertilisation. An inverse correlation was observed for the two parameters. Data from Tables 5.1 and 5.2 combined in this graphical representation.

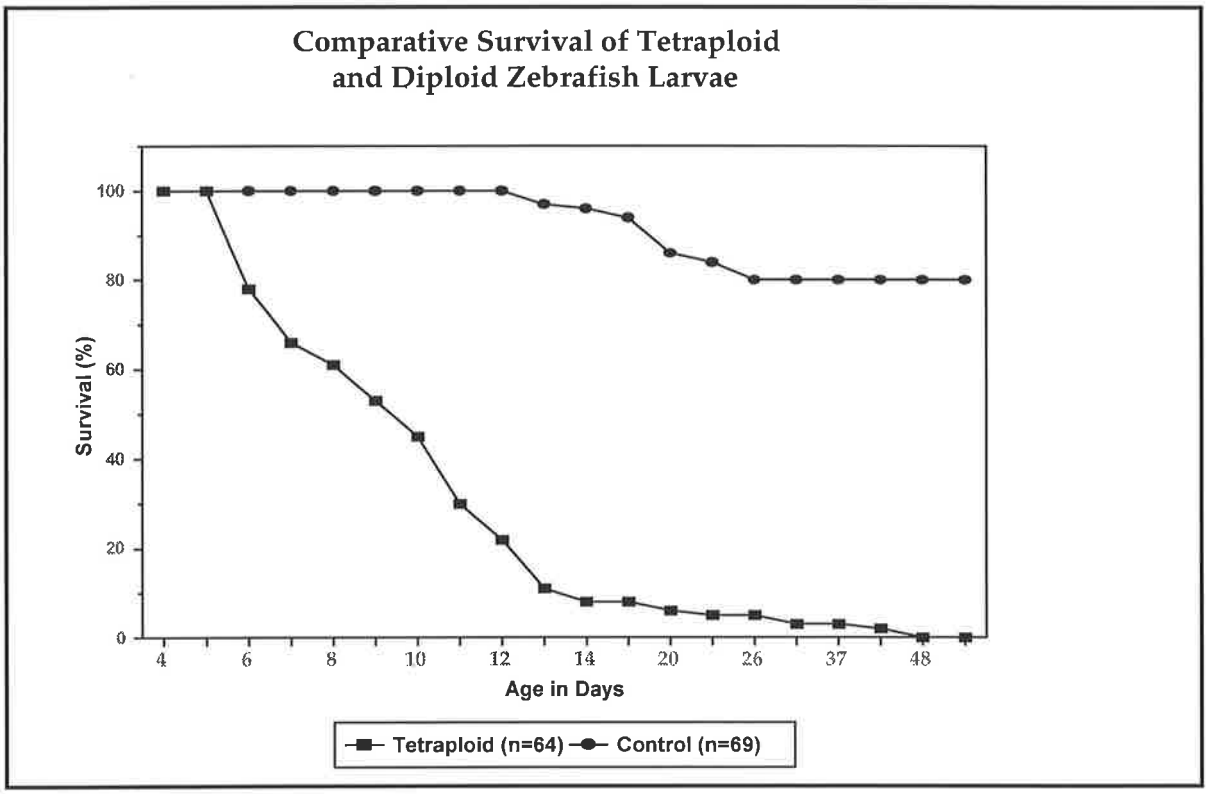


Figure 5.2 Survival of tetraploid zebrafish.
Tetraploid zebrafish larvae produced by HS treatment were raised by standard methods. Diploid larvae (control) that were not exposed to the HS were raised as controls

Control 24h zebrafish embryo

Typical zebrafish embryo at 24h that had not been exposed to heat shock (HS) treatment

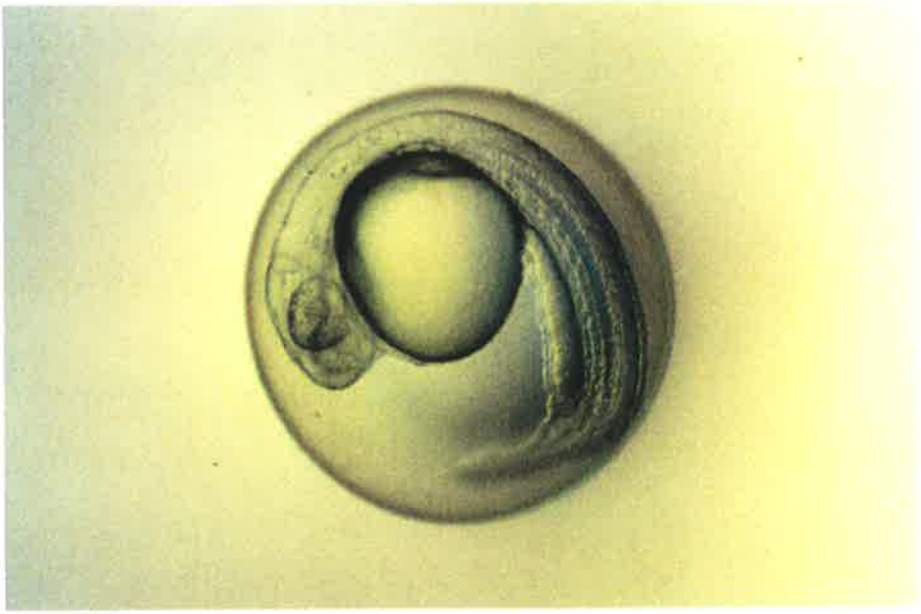
Morphologically normal tetraploid embryo at 24h

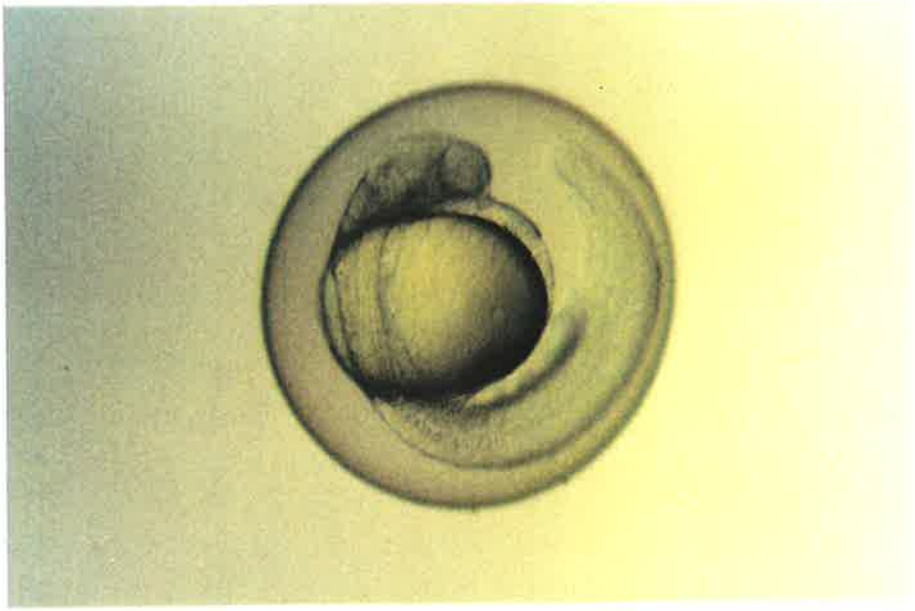
A tetraploid (4N) embryo with no apparent deformity, though the development of 4N embryos appeared slower than that of control embryos of comparable age

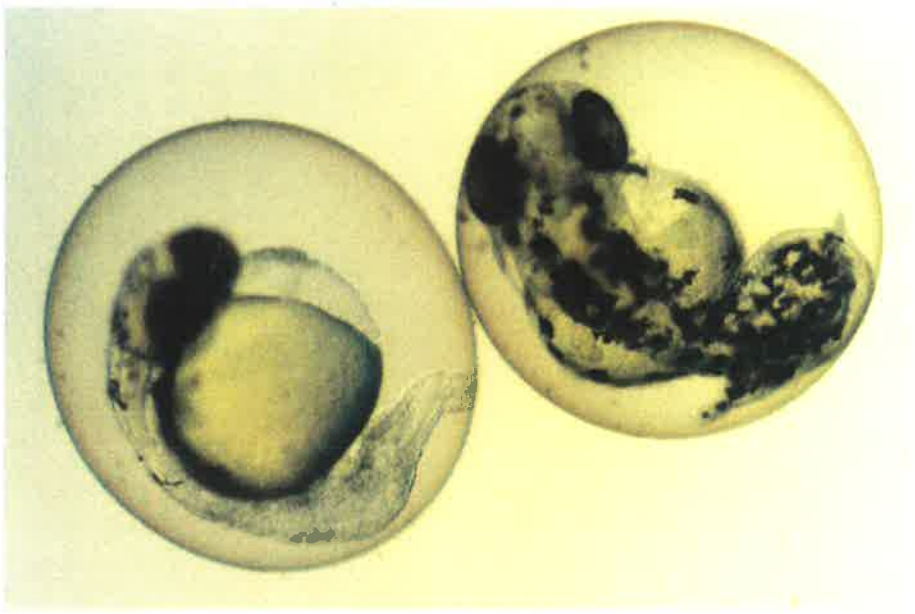
Deformed 48h old tetraploid embryos

Induced tetraploidy results in developmental abnormalities in some embryos, with spinal curvature, enlarged heart cavities and darkly pigmented phenotypes being the most common deformities observed

Plate 5.1 Zebrafish embryos resulting after HS induced tetraploidy







CHAPTER 6
GENERAL DISCUSSION

The potential for transgene technology to revolutionise aquaculture and piscine research is widely accepted. However there are several experimental, ethical and biohazard issues which need attention, before broad commercial acceptance of the technology is achieved. This thesis is primarily concerned with development of a laboratory model to explore ways and means of increasing the efficiency of producing transgenic fish.

The small Cyprinid species chosen *D. rerio*, commonly called zebrafish, has proven to be an extremely useful model for a wide range of research and biotechnical applications. Its attributes, which include genetic malleability and high fecundity, with clear, large, synchronously cleaving embryos being produced all year round, have been highlighted in this thesis.

The factors which limit its use for transgenesis studies include problems such as the high incidence of mosaicism following zygote injection resulting in a lower frequency of germline transgenics, and the lack of suitable manipulation techniques to allow treatment and analysis of large numbers of embryos. The development of procedures for injecting embryos through the chorion, instead of dechorionating them both simplified the microinjection procedure and improved survival of embryos resulting in a doubling of embryos survival compared with previous studies (45% in the present study compared with 16 and 26% Stuart *et al.*, 1988; and Culp *et al.*, 1991, respectively).

Transgenes containing DNA sequences of bacterial origin (*lacZ*, ***cat***) were found to present analytical problems relating to background expression due to the unexpected occurrence of bacteria-like inclusions in the gut, which complicated interpretation of the data. This problem was offset by the use of transgenes

incorporating the luciferase (***luc***) sequence isolated from firefly (*Photinus pyralis*). The ***luc*** construct also had the advantage that the expression assay for it was cheap, sensitive and relatively rapid to perform, making large scale screening of putative transgenics feasible. As reported it proved possible to determine transgene expression in zebrafish embryos as early as 5h after fertilisation, which proved extremely useful in exploring different experimental approaches.

One significant finding is that microinjection of ovulated (metaphase II) oocytes, as opposed to zygotes, doubled the incidence of transgenesis, as identified in fin samples of adult founder (F0) fish (47 and 25% respectively). More importantly this novel approach dramatically ($P<0.001$) reduced the incidence of mosaicism, as nearly all the germline positive F0 individuals (5/6) transmitted the transgene in the expected Mendelian manner *ie*, to approximately half of their progeny. By contrast the transmission rates of germline transgenic founders resulting from zygote injection ranged from 7-17% (ave 12%), in the current study and was similar in other studies with the same species (Stuart *et al.*, 1988; 1990; Culp *et al.*, 1991).

A major factor contributing to this outcome was the discovery of factors, present in seminal fluid, which prevented the parthenogenetic activation of zebrafish oocytes, allowing a degree of control over the timing of microinjection.

Thus in the case of the oocyte injection protocol, analysis of fin clips of putative transgenic fish provided a reliable indication of the likelihood of the fish being germline transformants, as virtually all the fin positive F0 individuals in this group transmitted the transgene to their F1 generation *via* their germline. In the present study a large proportion of the fin positive F0 resulting from the zygote injection group also proved to be germline positive (6/9). Which contrasts with

other studies (Stuart *et al.*, 1988; 1990; Khoo *et al.*, 1992; Patil *et al.*, 1994), where only a small proportion (ranging from 0-25%) of fin positive F0 fish passed the transgene on to their progeny. This discrepancy could be explained, in part due to the use of linear as opposed to circular (or supercoiled) transgene constructs and the conduction of microinjection only at the one-cell stage to minimise the occurrence of mosaicism resulting from injection at later stages.

Successful sperm mediated transgenesis was also achieved in both zebrafish and medaka. A similar frequency of zebrafish retained the transgene as shown by studies on embryos and fin clips of adults (14 and 13%, respectively). Germline transgenic zebrafish resulting from sperm mediated transfer, passed the gene to their progeny at a low frequency ranging from 10-18%, however the efficacy of this technique appeared to vary between the two species with DNA transfer rates, of 14 and 36% observed for the larvae of zebrafish and medaka respectively. The ability to treat large numbers of embryos using this techniques would hold an appeal for its potential to be adapted to many other species, however further study will be required to optimise the protocols for each species.

The suitability of the Japanese rice fish (medaka) for transgenesis studies using sperm mediated DNA transfer deserves emphasis. The high rate of sperm mediated transfer, was achieved even though the sperm were exposed to comparatively low concentrations of DNA (10 μg/ml compared with 200 μg/ml used for zebrafish). However the present series of experiments also identify the limitations presented by this species. In particular the unique reproductive behaviour of medaka which does not allow easy transfer of technology developed with this species, to other species. The low fecundity of this species also proved limiting, and the need to sacrifice the broodstock to collect gametes would make it unsuitable for a number of applications. The requirement of low

temperature facilities to house medaka if long term survival is required, is an added problem in tropical countries.

The challenge to the experimentalist to develop ES cell technology for fish thus permitting site-specific gene transfer, insertional mutagenesis (gene targeting) or gene ablation is clearly identified. Putative ES cells have only recently been isolated for medaka (Ozato *et al.*, 1995) and preliminary research is promising for other species (Collodi *et al.*, 1992). The production of chimeras is an important step in developing the technology for zebra fish and the production of adult phenotypic chimeras at a frequency of 40%, following transfer of blastomeres is very encouraging. The fact that cell transplants were made between embryos belonging to different species is unique as previous studies have used phenotypic mutants of the same species. The demonstration that pigmentation can be used as a convenient marker to identify chimeras both at larval and adult stages, is useful for researchers interested in the zebrafish.

One observation made which is highly salient to the success of chimera formation, was the differences in cleavage rates displayed by embryos belonging to the two species used and the effect this had on chimera formation, and this would need to be taken into account when considering the use of different species for such studies.

Many of the biohazard concerns about transgenesis would be offset if reliable control of reproduction of genetically manipulated organisms could be achieved. The work reported by Chourrout *et al.*, (1986b) and Myers *et al.*, (1986b), on ploidy offers one possible avenue. These researchers demonstrated that triploid salmonids produced as a result of a 4N-2N cross, were sterile and showed increased rates of growth over diploid individuals. In this study induction of tetraploid zebrafish was attempted using temperature shock with preliminary work indicating only a limited efficacy of cold shock treatments. It

was demonstrated that heat shock (HS) treatments could be successfully employed to produce tetraploid zebrafish larvae.

Previously a major limiting factor in optimising treatments aimed at induction of tetraploidy has been the inability to identify tetraploids early in their development. Thus the novel non-invasive procedure developed in the present study based on cleavage studies which allowed identification of tetraploids during early development with 100% accuracy, is a significant advance. The availability of this simple procedure allowed investigations using over 6000 embryos as only the individuals in whom tetraploidy had been induced needed to be examined further. This is in marked contrast to previous studies where the inability to identify tetraploid individuals early in development has led to all the treated embryos being raised, therefore limiting the number of tetraploids that could be examined. The precise definition of a HS regime and the adaption of simple heating procedures allowed nearly 50% of embryos to be successfully treated (*ie* resulting in tetraploidy). However despite optimising the HS treatments, no 4N zebrafish embryos survived beyond 48 days of age and most individuals were sub-vital when compared to diploid fish with major morbidity due to deformities of the cardio-vascular system and spine.

Studies conducted at the Institute of Neuroscience, The University of Oregon, (Eugene, USA) aimed at producing haploid zebrafish embryos to aid genetic screening, have shown that a few, but only very few individuals produce gametes suitable for the production of haploids, whereas the gametes from the majority fail to produce proper haploid embryos (Kate Whitlock, per comm). Factors such as these and other treatments such as pressure shock need to be investigated further.

Production of 4N-2N chimeras using 4N embryos as a source of donor blastomeres could provide an alternative strategy to producing triploid

offspring, if 4N fish prove to be subvital. The reverse manipulation, using 4N embryos as developmentally retarded recipients, could increase the efficiency of colonisation by the donor blastomeres, resulting in increased rates of production of chimeras.

The overall success of the study was critically dependent on the ability to successfully rear larvae. Of primary importance in this process is the ability to produce sufficient quantities of live food of a size appropriate to the stage of development of the growing larvae. Paramecia have been identified as a suitable starter food for zebrafish larvae, and methods to produce relatively clean cultures in abundant quantities, using simple techniques have been devised. Using methods described in this thesis survival of wild type zebrafish embryos to maturity, in the range of 80-90%, can be routinely achieved.

The data obtained in these series of studies, confirmed the zebrafish as a very useful and adaptable low cost piscine model for studying vertebrate developmental biology and all aspects of biotechnology including transgenesis. This is exemplified by the present study in which the investigator was able to carry out the experimental work described and carrying the responsibilities of maintaining the fish stock and rearing the live food and larvae with a minimum budget and whilst working without technical assistance.

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