CENTRE FOR RESEARCH ON INTRODUCED MARINE PESTS

REVIEW AND EVALUATION OF THE POTENTIAL OF MOLECULAR APPROACHES FOR THE ENVIRONMENTALLY BENIGN MANAGEMENT OF THE COMMON CARP (*CYPRINUS CARPIO*) IN AUSTRALIAN WATERS

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CONTENTS

1. SUMMARY

This report reviews a number of different molecular approaches for control, and possible eradication of carp populations. These approaches include chromosomal manipulation, gender manipulation (via hormones and transgenic methods) and the introduction of inducible fatality genes via transgenic methods.

Chromosomal methodologies appear to offer only short term control of carp populations. Altering sex ratios can also produce short term benefits; however, population models indicate that using this approach XX females persist in the population, although at reduced absolute numbers, so that eradication is not possible. Consequently, neither chromosomal nor gender manipulation strategies appear to constitute a means of locally eradicating carp, though the techniques may still be useful as a means of augmenting other approaches.

In contrast, there appears to be scope for control and possible eradication through transgenic incorporation of an inducible fatality gene (IFG) into the carp genome. The expression of this lethal gene could be controlled by an inducible promoter and regulated by, for example, dietary supplements. The IFG itself could be introduced into a wild-type population by aggressive stocking of transgenic hatchery fish. Models suggest extensive penetration of the transgene into the target population after several generations, with the rate of introgression dependent upon the annual stocking rate relative to such variables as the rate of natural mortality of non-hatchery juveniles. In theory, the rate of introgression of the transgene into the target population can also be accelerated by manipulation of rearing techniques and, possibly, through use of introducing multiple copies of the IFG in each stocked individual. At an appropriate time, the IFG would be induced by the appropriate trigger (e.g., dietary supplement), which would cull all fish carrying the transgene with no impact on non-transgenic individuals (i.e., species other than carp). A combination of intermittent culling and re-stocking with transgenic fish appears likely to result in eventual eradication of carp from the target system.

Integrating a genetic weakness into wild populations via the stocking of transgenically modified fish appears to be a viable, if perhaps long term strategy for the control, and possible eradication of the carp from Australian waters. Furthermore, development of a broodstock of fish containing the IFG appears to be technically feasible given the current state of molecular biology.

2. INTRODUCTION

The common carp (*Cyprinus carpio*) is a 'coarse' fish, endemic to Asia but now spread nearly world-wide due to its value in small-pond aquaculture. The species was first introduced into Australian waters in the mid 1800s, but only attained 'noxious fish' status in the 1960s following the release of 'Boolara' strain fish into Victoria (McDowell, 1966). Originally introduced for aquaculture purposes, Boolara strain fish escaped into the Murray-Darling River system, where it is now estimated to constitute the overwhelming majority of fish biomass. Detailed studies of carp impacts are still in progress, but there is clear anecdotal information that indicate the species has had a major role in degradation of habitat quality and negative impacts on native fishes in the river systems it has colonized. In the 1990s, carp were also discovered in Lakes Crescent and Sorell, in Tasmania, raising widespread concerns about impacts on native fishes, water quality and recreational trout fishing. These concerns have increased efforts to find methods to control, if not eradicate, carp in Australia.

Historically, a variety of methods has been suggested to control carp, ranging from commercial harvesting to selective poisoning (see McCrimmon, 1968, Morison, 1989). Recent efforts in Australia, and in particular Tasmania, have focused on prospects for physical removal via fishing (netting and electro-shocking) and poisoning (through the application of rotenone). Unfortunately, none of these techniques is likely be completely effective, due to the difficulty of catching carp, their resistance to most fish toxicants (e.g., rotenone) and their high reproductive capacity. Overseas experience indicates that, given fecundities in the tens of thousands of eggs per female, missing even a small number of carp after applying conventional control options results in a rapid re-building of population numbers, to densities similar to that prior to control attempts (McCrimmon, 1968).

In the late 1970s and early 1980s, investigations started into alternative strategies for the eradication and/or control of carp. These strategies included biological control based on the introduction of carp-specific viral diseases or genetic manipulation. The former was based on a then seemingly promising rhabdovirus, known as spring viraemia. However, initial tests revealed, first, that the disease is not carp specific (it also infects, for example, such diverse fishes as pike and guppies), and secondly, that a significant proportion of carp was unaffected by the virus at temperatures above 18°C. Due to cost considerations and to obvious problems with specificity, research into the use of spring viraemia was abandoned in the early 80's after three years of investigation. Initial investigations into genetic approaches also suggested little scope for optimism. Although carp are well studied from a genetic perspective (see review by Wohlfarth, 1995), the then available technology suggested genetic manipulations would only be effective in small, confined populations, and that the chromosomal manipulations required to create generations of sterile offspring were well beyond the current state of technology.

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This report re-examines the potential of genetic manipulation for control/eradication of carp in the light of recent developments in molecular biology. This re-examination was prompted by both recently renewed concern of the impacts of carp on Australia's wild freshwater ecosystems and fisheries, and by a continuing perception that genetic manipulation has considerable logistical and safety advantages over conventional pest control methods (e.g., poisoning) and over pathogen-based approaches (e.g., viral diseases). Genetic manipulation is inherently species-specific; it can only involve the target organism, as opposed to other approaches which can be very non-specific. Inevitably, use of rotenone to clear a lake of carp, even if possible, means all other fish die, whereas a genetic approach could reduce carp numbers, and even eradicate the species, while leaving the rest of the system intact. And unlike natural pathogens, there is also no prospect for developed immunity to a genetic approach. If a pathogen does not kill 100% of the target species, it can result in the development of resistant individuals, which then limits the effectiveness of subsequent disease applications. By contrast, genetic weaknesses introduced through transgenic manipulation cannot be removed, eliminating any potential for developed immunity.

Two approaches were taken in this review. First, recent literature on genetic manipulations in fishes was reviewed, focusing on those which showed promise of application in a biological control context. The information from this review and the results of discussions with leading geneticists in Australia and overseas were combined, to produce a 'state-of-the-art' summation of current molecular techniques applicable to the problem of carp control. Second, the likely impact and effectiveness of genetic manipulation was tested by means of simple genetic models. These provide an assessment of the rate and scale of incorporation of genetic weaknesses into a target population, given a range of simplified assumptions.

3. RESULTS

3.1 TECHNIQUES FOR INSERTING TRANSGENE CONSTRUCTS INTO CARP EMBRYOS

Microinjection

Microinjection is one many standard techniques developed for transforming embryos. It typically involves a finely drawn out needle which is used to inject the transgene construct directly into the pronucleus or cytoplasm of a fertilized egg. However, the procedure is time consuming which limits the number of embryos that can be processed while still at the same, optimal, developmental stage. This method also alters the microenvironment within the egg and may affect their viability (Zhao *et al*, 1993).

Microinjection is also not applicable to all species. Typically, injection is made with a micropipette through the chorion or the micropyle of the fertilized egg. In some species, however, the chorion hardens quickly after fertilization, to a

thickness that will not allow the use of a micropipette. This problem can be minimized by the use of incubation media which delay hardening of the chorion (Knibb *et al*, 1994) or by piercing the chorion with a sharp piece of glass (Houdebine and Chourrout, 1991).

Retroviral Infection

Another approach to creating transgenic animals involves the use of retroviral infection. This technique has been avoided as a serious option due to the possibility of the retroviruses recombining with dormant viruses in the recipient's genome, causing cancers and other diseases which may not be restricted to the target species (Kolberg, 1992; Anderson, 1992; Zhao *et al*, 1993).

Electric Field Transfers

Because fish embryos divide quickly, a rapid and simultaneous transfer is imperative, so that all eggs are at the same stage of development when the transfer of DNA takes place. The use of electric fields permits the simultaneous transfer of DNA molecules into a large number of target fish eggs. By applying an electric current to a solution containing developing embryos, the DNA construct is electroporated into the cell. However, problems associated with the capacitor based systems typically used to generate the electric field can result in high death rate of treated samples, and irreproducibility of transfer efficiency. This has led to development of a system termed Baeckonization, which generates an electric field using noncapacitor electronic circuitry and has a noncontact mode of operation (Zhao *et al.*, 1993). The design of this system eliminates the possibility of electrocution and contamination. This method has been shown to be effective in the zebrafish, in which the transgenic DNA was passed onto the next generation and expressed its expected function in the transgenic fish.

Biolistic/Gene Gun

In 1987, Klein and others described a novel approach that enabled nucleic acids to be delivered into plant cells using high velocity microprojectiles. The microprojectiles used are small spherical particles of tungsten or gold, approximately four microns in diameter. The particles are coated with RNA or DNA and then accelerated at high velocities towards the target tissue. Once embedded in the cell, the RNA or DNA fragments dissolve into the cytoplasm and are subsequently integrated into the genome.

Enhancement of Gene Insertion

One of the problems associated with gene transfer is the limited number of individuals from which the DNA construct is inherited in a Mendelian fashion.

Typically, many individuals exhibiting expression are mosaics for the transgene, with only a few having it incorporated into germ line cells enabling stable inheritance (Ivics et al. 1993). Transformation into germline cells can be enhanced by injecting the transgene into first or second stage embryos.

Concentration of the DNA construct also affects integration success. Too much DNA results in reduced embryo survival; too little reduces the number of fish into which the foreign DNA has been successfully inserted. The optimal amount no doubt varies with both the target species and the gene of choice. In trout, up to 20nl, containing 200pg of DNA, is injected into the cytoplasm of the embryo. This corresponds to 50 million copies of the target gene (Houdebine and Chourrout, 1991). There are no data on optimal concentrations for carp.

Another method of enhancing insertion of the transgene involves the use of viral integration proteins. Ivics *et al.* (1993) demonstrated enhanced integration using the Moloney murine leukemiavirus (MoMLV) integration protein (IN) in embryonic zebrafish cells. Two forms of IN were tested and both were able to enhance expression in the zebrafish 10 days after fertilization. After 14 days those fish which had been injected with the IN had significantly higher levels of transgenic DNA than control fish (Ivics *et al*, 1993).

The integrase protein accomplishes the integration reaction in three steps:

1. recognition of the integrase protein recognition sequences (INRSs) and removal of two nucleotides from the 3' ends;

2. cleavage of the chromosomal DNA: and,

3. joining of the viral cDNA into the host chromosome.

Results showed that Moloney murine leukemia virus integration protein from two different sources, a baculovirus-insect cell expression system (BV) and the retrovirus packaging cell line -2, enhanced expression and integration in zebrafish following their co-injection with suitable reporter substrates into early embryos.

Ten days after microinjection the expression of the transgene was 40-fold higher in samples injected with -2 cell extract and 14-fold higher with BV/IN than in control embryos injected with either DNA or DNA plus LMT- cell extract. This suggests that more copies of microinjected DNA were being stabilized in chromatin as a result of IN activity (Ivics *et al*, 1993). Using this IN, enhanced integration of the construct into the chromosomes appears to be possible for at least some of the P1 generation and would thus speed the process of producing a line of fish which were homozygous for the new gene.

Identification of transgenic individuals

Detection of successful transformation can be accomplished through direct methods using polymerase chain reaction (PCR) or southern blotting techniques.

However, it is usually accomplished via a reporter gene system. These systems are included in the DNA construct and produce a detectable substance indicating successful integration.

Reporter genes contain an enzyme not normally present in the species of interest and possess an activity that is easy to assay. Several systems are commercially available, including luciferase (*Luc*), *Cat*, *LacZ*, and *ampr*. Recent trials have examined the effectiveness of these reporter genes both without a promoter as well as when linked to the promoter/enhancer of the long-terminal repeat (LTR) of the Rous Sarcoma Virus (RSV). These trials demonstrated that the *Luc* reporter gene system was superior to the other systems when used in fish cells and fish embryos (Sekkali et al. 1994).

3.2. MODELING THE RATE OF INTROGRESSION OF THE TRANS-GENE INTO THE WILD POPULATION

A model was developed to describe a theoretical rate of introgression of the transgene into a wild population. The model was developed to work as a simple Excel spreadsheet (Appendix I). For simplicity, the model assumed: i) a population which was at carrying capacity; ii) generations of individuals were discrete and non-overlapping; iii) equal reproductive capacity for all females (both wild and transgenic); and iv) uniform mortality across all individuals. The population is assumed to be in a closed system (e.g., a large dam or isolated section of a river) and the carrying capacity was set at 100,000 adult fish. Since up to 2.2 million eggs have been observed for an 851mm (10.1kg) female carp (Swee and McCrimmon, 1966), the value of two million was chosen as the fecundity of each female in the model population. The value of stocking density in Appendix I was set at 10% of the juveniles present in the wild at the age of stocking.

The numbers of fish (juveniles and adults) present in the population were calculated from fixed values. Fry mortality (= the survival from egg to age one juveniles) was arbitrarily set at 0.001, to reflect mortality in the wild up to the age at which hatchery fish were stocked. Juvenile mortality (= age one to mature adult) was calculated as the proportion required to reduce juvenile numbers to that of the adult carrying capacity; specifically, in this model juvenile mortality equals the number of juveniles (feral plus stocked) divided by 100,000. The relative abundance of different genotypes in the adult population was calculated from genotype frequencies among all juveniles and the juvenile mortality rate.

In the initial generation (F_0) only four genotypes were present: male and female wild-type and transgenic fish. The proportions and the total number of gametes produced were derived from the frequencies of adult genotypes. Since random mating was assumed, gamete frequencies based on adult genotype frequencies were placed into a Punnett square (in Appendix 1, the shaded area next to each potential gamete type), which permitted the calculation of F_1 feral genotype

frequencies. These frequencies were multiplied by the proportion of fry surviving to age one to obtain absolute numbers of juveniles (values under shaded area labeled "feral genotypes") present at the time hatchery fish were stocked into the system. Lastly, the number of adults surviving to breed was calculated by multiplying the numbers of juveniles of each genotype by the juvenile mortality value. These genotypes were then used to calculate the frequencies of gametes produced that would contribute to the F_2 generation. The process was then iterated to simulate several generations of stocking effort.

The three variables that could be adjusted in the simulation were i) carrying capacity, ii) eggs per female and iii) numbers of fish stocked. However, the impact of varying stocking rates is best visualized as a proportion of total reproduction, rather than as the absolute number of fish that can be stocked into a system. Representing the impacts in terms of proportional reproduction by transgenic and wild-type fish incorporates the effects of all three variables (ie. carrying capacity, eggs per female, and fish stocked), such that only stocking density needs to be adjusted to demonstrate the time required to reach 100% introgression of the transgene in the wild population.

Model results at six annual stocking rates are shown in Figure 1. Stocking rate clearly has a huge impact on the rate of transgene introgression. Achieving adult populations that are 50% transgenic requires many more than 50 generations at stocking rates less than 1%, but only 1-2 generations at a stocking rate of 20%. The largest change in the rate of transgenic introgression is between stocking rates of about 1 and about 10%.

To translate this into actual numbers of juveniles required for annual stocking, assume a carrying capacity of 100,000 adults, a 1:1 sex ratio and a mean fecundity of two million eggs per female. Hence, each year 10^{11} eggs would be produced, resulting in 100 million feral juveniles at age 1 (obviously dependent on rates of fry mortality). Thus, for transgenic fish to constitute 10% of the juvenile population at age one, 10 million transgenic juveniles would need to be stocked annually.

These results are indicative only, and crucially dependent on a number of assumptions about fecundity, stock-recruitment relationships and, in particular, rates of fry and juvenile mortality. In order to obtain more accurate time estimates and to help determine optimal stocking strategies, a more comprehensive model needs to be developed that uses realistic estimates of population parameters. The model is also genetically simple, in that it deals simplistically with integration of a single gene stocked into a wild population. Although not simulated here (due to model complexity), intuitively, incorporation rates of a transgene would be multiplied many times if stocked fish carried multiple copies of the transgene at several chromosome locations.

3.3 TYPES OF SEX MANIPULATIONS

Chromosomal manipulation of sex determination in carp

Sex determination in fish (teleosts) is accomplished through a wide variety of mechanisms (Yamamoto 1969). In the carp, the sex determining switch is thought to be genic and male dominant (XX, XY) since sex ratios in conventional diploid or induced triploid offspring approximate a 1:1 ratio and gynogenetically produced offspring are normally all female (Nagy and Csanyi, 1984; Wu *et al.*, 1990; Komen *et al.*, 1991).

Two techniques have proven useful for manipulating sex determination in carp. These include hormonal sex reversal, chromosomal manipulation (Nagy *et al.*, 1981; Komen *et al.*, 1991). Chromosomal manipulation involves elimination of either paternal (gynogenesis) or maternal (androgenesis) chromosomal DNA. Gynogenesis begins with the fertilization of eggs using sterile sperm. The diploid state of the egg is returned to the zygote following fertilization by treating the eggs with either a temperature or high pressure shock to block extrusion of the polar body (heterozygous gynogen) or to block the first mitotic division (homozygous gynogen). All resulting individuals are female (XX). Androgenesis involves the use of gama rays to destroy maternal nuclear DNA in unfertilized eggs. After fertilization of these eggs with normal sperm, a pressure shock is applied at the appropriate time to block first polar body extrusion to return the zygote to a diploid state. Resulting individuals are completely homozygous at all loci and either XX or YY with respect to their sex chromosomes.

Hormone induced sex determination typically involves the use of androgens to induce production of males in XX females. Male sex inversion (XX males) can be achieved by the administration of androgens prior to phenotypic expression of gonadal sex (Komen and Richter, 1993). However, application of hormones does not guarantee 100% conversion and precise amounts of hormone are required as too little results in a mixed sex populations while too much can cause sterilization.

Testosterone appears to be the steroid of choice for sex reversing females. Sex inversion trials conducted using the synthetic androgen analogue, 17-alpha-methyltestosterone (17-a-MT) involve administration to young fish as a dietary supplement. The most successful period of application appears to be from 6-11 weeks post-hatch (Komen and Richter, 1993). This trial resulted in >90% males with very low proportion of females and sterile fish. Administration at 3-8 weeks post-hatch resulted in production of 7% males and 93% sterile fish. A third trial period involved administration of the hormone at 10-15 weeks post-hatch. This trial also resulted in more males than the expected 50%, but females were still present in the cohort as well as sterile and intersex fish. Basavaraja and Satyanarayana (1988) administered 17-a-MT to one day old fry for periods

of 30 and 50 days at varying concentrations in order to produce sterile populations of carp. This proved to be effective for the longer trial period with 20 males and 120 sterile fish being produced after the growout period of 255 days. From these examples, that the administration of the hormone during the first 6 or 8 weeks after hatching would seem more likely to result in sterile rather than male fish. The most effective period of administration would therefore appear to be closer to the range of 6 to 11 weeks if Komen and Richter's (1993) work is followed. While sterility is not a desirable trait for long term eradication, it may provide containment methods for preliminary release trials of transgenically modified fish.

Mibolerone is another hormone tested as an oral inducer of sex reversal in teleosts (Das *et al.*, 1990; Sobhnana and Nandeesha, 1994). This androgen was however tested over the same period in both studies, beginning at day 3 after hatching and extending to 30 days. All trials of mibolerone resulted in a high percentage of sterile compared to male fish, but this could be due to the time of administration as suggested by results of 17-a-M.

In addition to duration of treatment, concentration of hormone is another factor affecting sex ratio and proportion of sterility among treated fish. The optimum concentration of the hormone on release can only be based on the findings of optimal concentrations of exogenous administration of these hormones in the diet of the fish. A major problem with determining the appropriate dose is the release of excess hormone as an excretory product and production by the fish's own endocrine system. Testosterone administered orally exhibits approximately one-sixth the activity of injected testosterone, due to partial inactivation by the liver. Basavaraja and Satyanarayana (1988) have performed sex reversal experiments involving varying concentrations of 17-a-MT in the food of the common carp. Female free populations were produced in trial periods of 30 days using concentrations of 400ppm and after 50 days using 200 ppm. The 50 day trial also showed that at higher concentrations for this length of time, the ratio of male fish to sterile fish was reduced, with the population being almost completely sterile at a concentration of 300 ppm (table 1).

Table 1 Effect of dietary administration of the androgen, 17-alpha-methyltestosterone on sex of common carp (After : Basavaraja and Satyanarayana, 1988).

Treatment	Males	Females	Sterile	Sex ratio Male:Female
Control expt.1 (30 days)	23	29	0	1 : 1.3
17-a-MT (200ppm)	3	5	9	1 : 1.7
17-a-MT (300ppm)	10	7	37	1 : 0.7
17-a-MT (400ppm)	7	0	11	7 : 0
Control expt.2 (50 days)	31	28	0	1 : 0.9
17-a-MT (200ppm)	20	0	120	20 : 0
17-a-MT (300ppm)	1	0	121	1 : 0

Dietary administration of 17-a-MT at a concentration of 100g/g for 36 days resulted in 83% male carp (Yarzombek and Gomel'skii, 1993). All masculinized fish matured as normal males after 8-9 months. In this experiment the population was >80% males with no mention of sterility problems. This value is much higher than in other trials of 17-a-MT which have used higher concentrations of the hormone in the diet for shorter trial periods. The concentration used in this example was 100ppm for a trial length of 36 days. This compares to concentrations of 400ppm for 30 days and 200 ppm for 50 days tested in other experiments, both leading to a majority of sterile fish in the populations. From the results it would seem possible to create a higher percentage of male fish by the administration of lower concentrations (<100ppm) for longer time periods (>36 days), this may reduce the chance of inducing sterility whilst increasing the percentage which becomes masculinized.

The effects of varying concentrations of mibolerone have also been investigated by a number of studies. After 30 days of dietary administration, at concentrations as low as 5mg/Kg, the resulting population was predominately sterile (95%) with the balance being males (Das *et al.*, 1990). The effect of increasing the concentration of mibolerone in the diet was an increase in the number of sterile fish compared to male fish, with no apparent effect on the mortality rates (Table 2). Increasing the concentration of mibolerone has been suggested to be conversely related to the growth rates of the fish but this study observed no such effect (Das *et al.*, 1990). 17-a-MT, however, has shown an increase in the growth rates of the fish with increasing concentration up to a threshold level at which survival began to decline (Basavaraja and Satyanarayana, 1988).

The minimum required dosage of mibolerone required to produce a female free population was determined to be 2 mg/Kg diet for a 30 day period (Das *et al.*,

1990; Sobhnana and Nandeesha, 1994). This regime produced 30% males and 70% sterile fish and no females.

Table 2 Effect of dietary administration of the androgen mibolerone on sex of common carp.

Treatment	Males	Females	Sterile	Sex ratio Male:Female
Control	30	26	0	1 : 0.9
Mibolerone (5ppm)	5	0	86	5 : 0
Mibolerone (10ppm)	1	0	85	1 : 0
Mibolerone (15ppm)	0	0	97	0 : 0

Production of YY male fish

Another method to produce all male populations of fish is to use a combination of hormones and selective breeding to create males with two copies of the male sex determining genes (Mair et al., 1995). Firstly, sex reversed (XY) females were produced by oral application of the estrogen diethylstilboestrol (DES) to sexually undifferentiated fry. These were crossed with normal (XY) male tilapia, then progeny tested to find novel YY males. Crossing these males (YY) with sex reversed (XY) females led to the production of more YY males. In *Tilapia*, these YY males have been shown to be as viable and fertile as their XY and XX counterparts. All male populations created in this manner exhibit faster growth rates and better survival than those female free populations created by hormone based techniques such as 17-a-MT, mibolerone and chromosomal manipulation techniques of androgenesis and gynogenesis. Their growth rates were even higher than those of mixed sex control populations.

This method of obtaining female free populations of fish would be useful only in culture conditions, as the F1 of YY males with XX females in the wild would produce normal XY males. Its effect would be eliminated in one generation, as the F1 fish would mate with XX females resulting in an equal sex ratio among the F2 fish. Continuous stocking would be required to ensure that there were still homozygous YY males present. However, these fish have been shown to grow faster than hormonally induced males and would thus provide a good vehicle for introducing a gene of interest into natural populations.

Unfortunately, the majority of work on producing YY males has been done with *Tilapia*. Despite successful experiments with sex inversion of females to males using 17-alpha-methyltestosterone, the conversion of males to females with 17-beta-estradiol showed no significant effect on sex ratios in carp. However, there may still be scope for experimentation in this area.

Speculation on Sex manipulation via introduced male modifier gene

Control of sex determination can be easily accomplished in teleosts using hormonal treatments. However, an alternative approach may be the introduction of genes which form part of a critical pathway involved in gonadal differentiation. Such manipulation or control relies on the ability to find a gene which codes for substances capable of causing sex reversal in the fish. Following discovery and understanding the mode of action, this gene could then be implanted using transgenic methodologies.

Genes exhibiting these desirable characteristics have been located in both the mouse and human genome. Associated genes have also been described in many other mammals as well as in the fruit fly, *Drosophila melanogaster* (Koopman *et al.*, 1991). In mammals, this gene has been associated with the central event in sex determination, in which the testes rather than ovaries differentiate from the indifferent gonad (gonadal ridge). All other differences between eutherian mammals are thought to be due to the hormones or factors produced by the gonads. For this reason, sex determination is equivalent to testis determination. The Y-chromosomal gene product(s) responsible has been named TDY (testis determining Y chromosome) in mice and TDF (testis-determining factor) in humans (Sinclair *et al.*, 1991).

The TDY gene appears to be quite effective for causing chromosomal females to differentiate as males. Following its isolation from the sex determining region of the human Y-chromosome (SRY), an SRY gene was identified as a testis determining gene (Sinclair *et al.*, 1990). Evidence has been presented that mutations in this gene can lead to XY sex reversal (Berta *et al.*, 1990; Jager *et al.*, 1990). In addition, subsequent experiments demonstrated male development in chromosomally female mouse embryos implanted with a transgene isolated from the SRY (Koopman *et al.*, 1991), definite proof for equating the SRY/Sry with the mammalian testis-determining factor.

Although no such gene has yet been described in the literature for any teleost, there are promising leads that may help to find one. Gene sequences resembling the mouse (Sry) and human (SRY) have been found in many other mammals, as well as in the fruit fly, *D. melanogaster* (Koopman *et al.*, 1991). Conserved regions between these sequences may permit the development of a heterologous probe to screen for this gene in carp. In addition, a recessive mutation has been described which causes chromosomally female carp to develop as functional males (Komen *at al.*, 1992a). This gene is thought to inactivate male sex determining genes during female sex differentiation (Komen *et al.*, 1992b) and could therefore be a serious candidate for use as a modifier gene. Once the gene has been cloned and its mechanism of action determined, it should offer a possible alternative procedure to hormonal control for manipulating male development in carp.

Hence, effective hormonal and genetic approaches can be used to alter carp sex ratios. However, modeling these approaches indicates that such alterations will have only short term benefits, by reducing the number of females producing eggs. This proportion is directly related to the total number of males stocked into the population, with continued stocking required to maintain the effect. Furthermore, huge numbers of fish would be required for stocking due to high individual fecundity and the fact that populations are most likely constrained by carrying capacity.

3.4. INDUCIBLE GENETIC FATALITY

The second genetic manipulation approach investigated for biological control of carp was the introduction into the target population of an inducible fatality gene (IFG) via transgenic methodologies. An IFG is a genetic construct which adds to the genome a physiological weakness that under normal circumstances is not expressed (i.e., the fish carrying it does not die immediately). The expression of the lethal gene is controlled by an inducible promoter, which is switched on when culling is desired. The inducible promoter used depends on the particular IFG used, but can be as simple as providing zinc as a dietary supplement.

Transgenic techniques could be used to incorporate the IFG and promoter into a population of hatchery fish, which would then be used as broodstock to produce juveniles carrying copies of the transgene. The juveniles are planted into the wild via an aggressive stocking campaign and interbreed with and thereby integrate the transgene into the wild population in a Mendelian fashion. After several generations, the stocking program results in all fish in the wild having at least one copy of the transgenic construct. At the appropriate time, the dietary supplement could be provided, which induces the promoter and, in turn, causes expression of the lethal gene product and culls all fish carrying the transgene. Intermittent periods of culling and stocking should ensure that eventually all carp are eliminated from the system.

Inducible fatality genes have not been used as a biological control agent, although the idea has been around for many years. Molecular techniques have only recently been developed to the point where an IFG now appears to be feasible. Promising developments have occurred both with regard to the inducible promoters and the lethal gene itself.

Regulation of the Inducible Fatality Gene

Control over the expression of the induced fatality gene (IFG) is required in order for the gene to become fully integrated in the population. If the gene expressed its function too early, then stocked fish would die prior to passing the gene on to the next generation. In effect, only hatchery fish would die, which prevents the spread of the genetic weakness to the whole population. In order

to maintain control over timing of activation of the gene, the appropriate regulatory mechanism must be incorporated into the transgene construct.

The process by which the expression of genes is turned on in response to a substance is called induction. Genes regulated in this manner are known as inducible genes and are regulated at the transcription level (ie. the stage just prior to synthesis of the gene product). In order for transcription of the gene to be initiated, an RNA polymerase molecule must first bind to a promoter site located upstream of the gene sequence. In an inducible gene, this promoter site is typically blocked by a repressor protein which sterically inhibits RNA polymerase from binding and initiating transcription. Whether or not the repressor binds to the promoter site depends on the presence of a metabolite, called an effector molecule. Once the effector molecule is present, it binds the repressor molecule, which allows the RNA polymerase to transcribe the gene. The gene product then performs its function.

A number of different promoters have been tested to regulate gene expression in transgenic fish, including the carp B-actin promoter (Lui et al., 1990), fish antifreeze protein promoter (Gong *et al.*, 1991), and metal inducible metallothionein promoters (Inoue *et al.*, 1992). Metallothionein genes have been cloned from a number of species, including human, mouse, and fish. The regulatory elements of these genes have been extensively examined as models for studying gene expression, and they have also been used as promoters in transgenic fish (Inoue *et al.*, 1992).

Metallothionein, in particular, is an excellent candidate to be used as an inducible promoter in carp, as it can regulate the IFG through a simple dietary supplement. The metallothionein gene produces low molecular weight proteins which function to bind heavy metals such as zinc, cadmium, and copper. Thus, the metallothionein gene promoter is activated in the presence of such heavy metals. Inoue *et al.* (1992) demonstrated that both mammalian (mouse mMT-I) and fish (rainbow trout, rtMT-A) metallothionein promoters could be used to successfully regulate gene expression in fish through the simple addition of zinc to their diet. Hence, one very real possibility is that an IFG in carp could be triggered by distributing food pellets containing a zinc additive; other fish in the area, lacking the transgene, would get a good feed, but be otherwise unaffected.

Fatality Gene Candidates

A variety of mechanisms have been proposed as having the potential to induce cell fatality. Poulsen *et al.* (1989) describe a family of genes as candidate cell-killing mechanisms in bacteria. The toxic lectin, ricin, is another promising candidate (for review see Olsnes and Sandvig, 1988). Ricin is a potent inhibitor of protein synthesis in eukaryotes, and is found in the seeds of *Ricinus communis* (Osborn and Hartley, 1990). It consists of two structurally and

functionally distinct polypeptides, the α and β chains (Olsnes and Pihl, 1982). The β chain is the lectin with a dual role of first binding to galactose residues on the cell surface and then assisting in transport of the α chain into the target cytoplasm. The α chain is an *N*-glycosidase and has been shown to have a cyto-toxic effect by inactivating ribosomes, causing a cessation of protein synthesis. The α chain appears to require the β chain in order to enter the cytoplasm. Thus, if a transgenic construct of DNA containing the ricin α chain (coupled with an inducible promoter) is transfected into fish embryos (i.e., inserted genetically without the β chain), upon promotion it could act as an effective IFG, killing the cells in which it is was present. However, even if ingested, the α chain present in these cells would not be fatal in other organisms due to the absence of the β chain.

4.   MANAGEMENT IMPLICATIONS AND RECOMMENDATIONS

Integrating a genetic weakness into wild populations via the stocking of transgenically modified fish containing an inducible fatality gene (IFG) appears to be a viable, if perhaps long term strategy for the environmentally benign control, and perhaps eradication of the carp from Australian waters. Development of a broodstock of fish containing the IFG appears to be technically feasible given the current state of molecular biology. Refinement of an optimal inducible promoter and development of an efficient delivery vector for the effector molecule are components of a successful system that require development. These are not trivial tasks, but they appear to be quite achievable, given the current state of technology. Development of an IFG system for carp could be done in 3 to 5 years. Integration into the wild population would then depend on ecological variables and the efficiency of stocking strategies.

The long term nature of developing and then integrating the transgene into a wild population prior to induction is perhaps less immediately appealing than currently applied methods, such as application of toxicants and physical removal, which produce rapid and highly visible results. However, such methods often have highly undesirable side-effects on other components of the ecosystem, and very often do not constitute a long-term solution. As well, a variety of techniques can potentially be used to accelerate the development and optimization of some components of an IFG-based approach. For example, there are well-established husbandry techniques for carp that could be used to substantially reduce the time taken for fixation of the IFG in brood stock, by reducing age at sexual maturity, by shifting sex ratios to, potentially, all male broods, and by increasing the size at stocking of transgenic fish relative to wild fish. Optimization of grow-out techniques is also likely to minimize developmental and stocking costs.

Time to full integration into the wild population could also be reduced by incorporating several IFG constructs in different chromosomal locations.

Stocking a fish that has 10 unlinked homozygous chromosome locations of the IFG would be as effective as stocking 10 fish, each with a single IFG construct. To an extent, creation of multiple carriers of the IFG is highly likely in any case during the initial development of the transfected fish, as the probability of integrating the construct in the exact same chromosome location in all individuals is very low. Inter-breeding of the transgenic brood fish would effectively achieve offspring with multiple copies, thereby accelerating incorporation into the wild target population relative to the rates suggested in Figure 1.

More realistic estimates of basic ecological and biological variables for carp than those used in the model would also streamline any stocking program and maximize IFG introgression. Variables such as carrying capacity of various age classes, timing of spawning, fecundity, age class structure, and mortality schedules will facilitate accurate modeling as well as assist with determining the feasibility of any stocking strategy.

5. REFERENCES

Basavaraja, N. and G. P. Satyanarayana. 1988. Induction of sterility in the common carp, *Cyprinus carpio* (Linn.) using 17-alpha-methyltestosterone. Indian Journal of Animal Sciences 58 (10): 1248-1251.

Das, S. K., H. P. C. Shetty, and M. C. Nandeesha. 1990. Production of female-free common carp, *Cyprinus carpio* var. *communis* (L.) through dietary administration of the androgen mibolerone. Asian Fisheries Science 3: 197-203

Houdebine , L. M. and D. Chourrout. 1991. Transgenesis in Fish. Experentia 47: 891-897.

Ivics, Z., Z. Izsvak, and P. B. Hackett. 1993. Enhanced incorporation of transgenic DNA in Zebrafish chromosomes by a retroviral integration protein. Molecular Marine Biology and Biotechnology. 2(3): 162-173.

Knibb, W., Elizur, A., Moav, B. and A. Robins. 1994. Inhibition of egg chorion hardening in the marine teleost, *Sparus aurata*. Molecular Marine Biology and Biotechnology. 3 (1): 23-29.

Komen, J., A. B. J. Bongers, C. J. J. Richter, W. B. van Muiswinkel and E. A. Huisman. 1991. Gynogenesis in common carp (*Cyprinus carpio* L.) II: the production of homozygous gynogenetic clones and F1 hybrids. Aquaculture, 92:127-142.

Komen, J. P., M. Yamashita and Y. Nagahama. 1992a. Testicular development induced by a recessive mutation during gonadal differentiation of female

common carp (*Cyprinus carpio*, L.). Developmental Growth and Differentiation. 34:535-544.

Komen, J., P de Boer and C. J. J. Richter. 1992b. Male sex reversal in gynogenetic XX females of common carp (*Cyprinus carpio*, L.) by a recessive mutation in a sex determining gene. Journal of Heredity. 83:431-434.

Komen, J. and C. J. Richter. 1993. Sex Control In Carp. Recent Advances in Aquaculture. 4:78-86.

Mair, G. C., J. S. Aubucay, J. A. Beardmore and D. O. F. Skibinski. 1995. Growth performance of genetically male tilapia (GMT) derived from YY-males in *Oreochromis niloticus* L. - on station comparisons with mixed sex and sex reversed male populations. Aquaculture 137: 313-323.

McCrimmon, H.R. 1968. Carp in Canada. Fisheries Research Board of Canada, Bulletin 165: 93 Pp.

McDowell, R. 1996. Freshwater fishes of South-eastern Australia. 2nd Ed. Reed, Sydney, Australia. 247 Pp.

Morison, A. K. 1989. Management of introduced species in the Murray-Darling basin - a discussion paper. Proceedings of the Workshop on Native Fish Management, Pp. 149-161. Murray-Darling Comm., Canberra, Australia.

Nagy, A., M. Bercsenyi and V. Csanyi. 1981. Sex reversal in carp (*C. carpio*) by oral administration of methyltestosterone. Canadian Journal of Fisheries and Aquatic Sciences. 38: 725-728.

Nagy, A. and V. Csanyi. 1984. A new breeding system using gynogenesis and sex reversal for fast inbreeding in carp. Theoretical and Applied Genetics. 67: 485-490.

Nikitina, M. M. and P. L. Godovich. 1983. Dynamics in the blood of male and female carp (Cyprinus carpio) during the spawning period. Journal of Ichthyology. 23:118-123.

Shears, M. A., G. L. Fletcher, C. L Hew, S. Gauthier, and P. L. Davies. 1994. Transfer, expression and stable inheritance of antifreeze protein genes in Atlantic Salmon (*Salmo salar*) Molecular Marine Biology and Biotechnology. (1994) 1 (1): 58-63.

Sekkali, B., Belayew, A., Martial, J. A., Hellemans, B. A., Ollevier, F. and F. A. Volckaert. 1994. Reporter gene activities in fish cells and embryos Molecular Marine Biology and Biotechnology. 3 (1): 30-34

Wohlfarth, G. W. 1995. The common carp and Chinese carp. In Conservation of Fish and Shellfish Resources - Managing Diversity (Eds., J. Thorpe, et al.), Pp. 138-170. Academic, New York.

Wu ,C. J, Chen, R. D., Ye, Y. Z. and W. Y. Huang. 1990. Production of all female carp and its application in fish cultivation. In Genetics in Aquaculture III. (Ed., Gjedrem,T.) 85:1-4..

Yamamoto, T. 1969. Sex differentiation. In Fish Physiology, Vol. 3 (Eds., W. S. Hoar and D. J. Randall), Pp.117-175. Academic, New York.

Yarzombek, A. A. and B. I. Gomel'skii. 1993. Solubilization of Methyltestosterone in Carp. Journal of Ichthyology, 33 (1): 147-148.

Zhao, X., P. J. Zhang, and T. K. Wong. 1993. Application of Baeckonization: a new approach to produce transgenic fish Molecular Marine Biology and Biotechnology. 2(1): 63-69

FIGURE 1: PROPORTION OF THE POPULATION CARRYING THE TRANSGENE AT VARYING STOCKING DENSITIES USED AS INPUT INTO THE MODEL (APPENDIX I). STOCKING DENSITIES WERE DEFINED AS A PERCENTAGE OF JUVENILES PRESENT IN THE POPULATION AT AGE ONE.
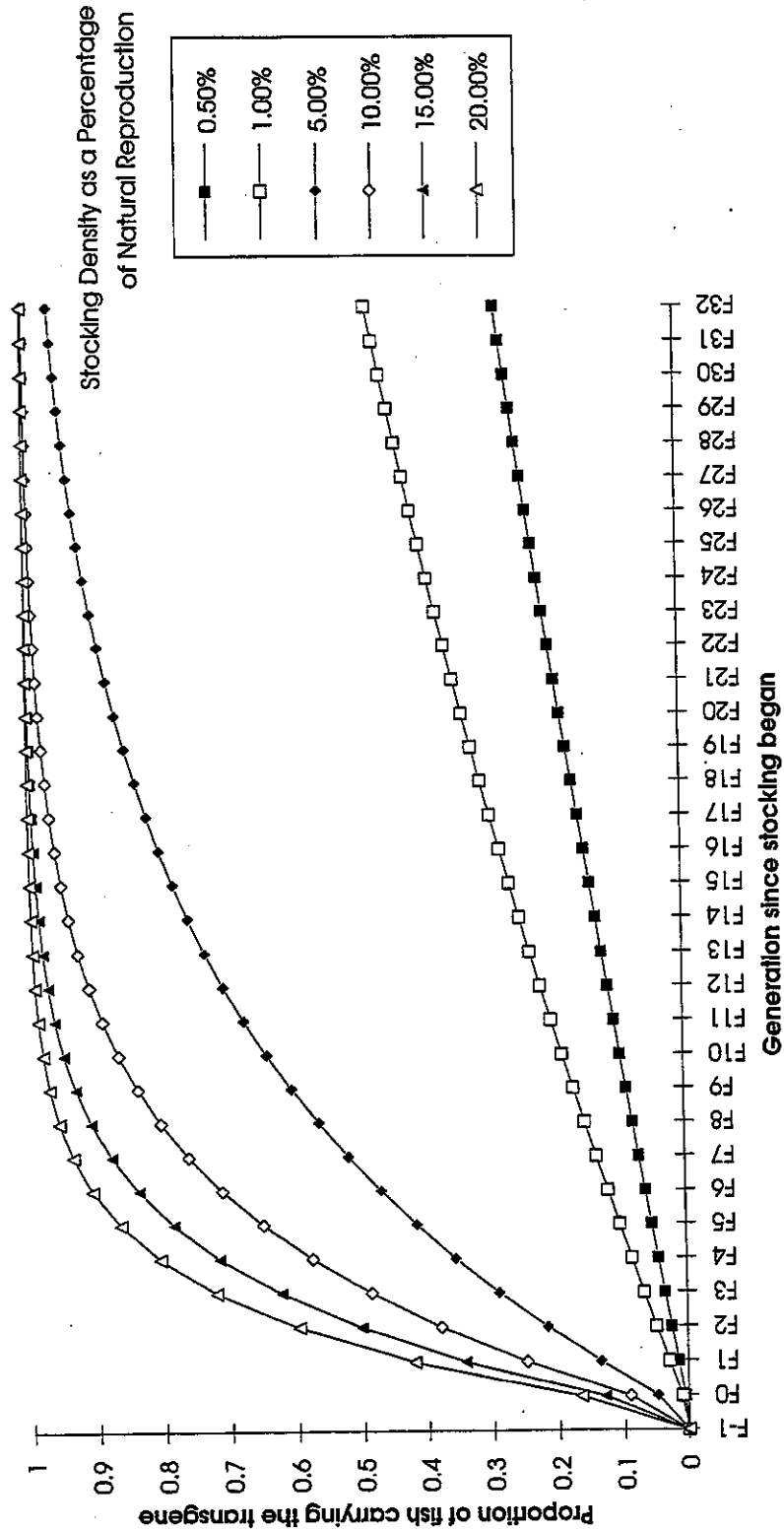
APPENDIX I: DETAILED SPREADSHEET DESCRIBING MODELING INTEGRATION OF A TRANSGENE INTRODUCED TO A POPULATION VIA STOCKING OF HATCHERY FISH.

Appendix I. Model demonstrating the rate of integration of a transgene introduced into the populaiton via the stocking of fish homozygous for the gene. Values for carrying capacity, eggs per female, juveniles surviving to age one, and stocking rates are in thousands of fish. Stocking begins at generation Fo. The number of feral juveniles surviving to age one assumes a survival rate of 1 in 1000.

carrying capacity=	100
eggs per female =	2,000
stocking rate =	10,000
mortality = carry.cap/(repro.cap+stock.cap)	

Fo

	feral genotypes			stocked genotypes			%
	XX	XY		XX^^	XY^^	total juvs	stocked
100,000 juvs surviving	50,000.	50,000.		5,000.	5,000.	110,000.	10%
mortality 0.0009091							
Adult genotypes	XX	XY		X^X^	XY^^	total adults	
Adults that survive	45.5	45.5		4.5	4.5	100	
adult freq.	0.45	0.45		0.05	0.05	1	

Punnett square M F	feral gametes				stocked gametes	
	x	y	x^	y^	x^	y^
	0.45455	0.454546455			0.05	0.05
0.91 x	0.41322	0.41322			0.04132	0.04132
0.00 x^						
0.09 x^	0.04132	0.04132			0.00413	0.00413

F1

	feral genotypes						stocked genotypes			%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	total juvs	Stocked
100,000 juvs surviving	41322.3	41322.3	8264.46	8264.46	413.223	413.223	5000	5000	110,000	10%
mortality 0.0009091										
Adult genotypes	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	total adults	
Adults that survive	37.57	37.57	7.51	7.51	0.38	0.38	4.55	4.55	100	
adult freq.	0.38	0.38	0.08	0.08	0.00	0.00	0.05	0.05	1	

25% of the adults contain the transgene

Punnett square M F	feral gametes				stkd gametes	
	x	y	x^	y^	x^	y^
	0.41322	0.41322	0.04132	0.04132	0.05	0.05
0.83 x	0.34151	0.34151	0.03415	0.03415	0.03757	0.03757
0.08 x^	0.03415	0.03415	0.00342	0.00342	0.00376	0.00376
0.09 x^	0.03757	0.03757	0.00376	0.00376	0.00413	0.00413

F2

	feral genotypes						stocked genotypes			%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	total juvs	stocked
100,000 juvs surviving	34150.7	34150.7	14343.3	14343.3	1506.04	1506.04	5000	5000	110,000	10%
mortality 0.0009091										
Adult genotypes	XX	XY	XX^	XY^	XX^^	XY^^	XX^X^	XY^^	total adults	
Adults that survive	31.0461	31.0461	13.0393	13.0393	1.36913	1.36913	4.54545	4.54545	100	
adult freq.	0.31046	0.31046	0.13039	0.13039	0.01369	0.01369	0.04545	0.04545	1	

35% of the adults contain the transgene

F3

| | feral genotypes | | | | | | stocked genotypes | | | % |
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	totals	stocked
100000 juvs surviving	28224	28224	18684	18684	3092	3092	5000	5000	110,000	10%
Adults that survive	25.66	25.66	16.99	16.99	2.81	2.81	4.55	4.55	100	
adult freq.	0.26	0.26	0.17	0.17	0.03	0.03	0.05	0.05	1	

49% of the adults contain the transgene

F4

| | feral genotypes | | | | | | stocked genotype | | 100,000 | % |
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	23325	23325	21651	21651	5024	5024	5000	5000	110,000	10%
Adults that survive	21.20	21.20	19.68	19.68	4.57	4.57	4.55	4.55	100	
adult freq.	0.21	0.21	0.20	0.20	0.05	0.05	0.05	0.05	1	

55% of the adults contain the transgene

F5

| | feral genotypes | | | | | | stocked genotype | | 100,000 | % |
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	19277	19277	23538	23538	7185	7185	5000	5000	110,000	10%
Adults that survive	17.52	17.52	21.40	21.40	6.53	6.53	4.55	4.55	100	
adult freq.	0.18	0.18	0.21	0.21	0.07	0.07	0.05	0.05	1	

60% of the adults contain the transgene

F6

| | feral genotypes | | | | | | stocked genotype | | 100,000 | % |
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	15932	15932	24584	24584	9484	9484	5000	5000	110,000	10%
Adults that survive	14.48	14.48	22.35	22.35	8.62	8.62	4.55	4.55	100	
adult freq.	0.14	0.14	0.22	0.22	0.09	0.09	0.05	0.05	1	

71% of the adults contain the transgene

F7

| | feral genotypes | | | | | | stocked genotype | | 100,000 | % |
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	13167	13167	24983	24983	11851	11851	5000	5000	110,000	10%
Adults that survive	11.97	11.97	22.71	22.71	10.77	10.77	4.55	4.55	100	
adult freq.	0.12	0.12	0.23	0.23	0.11	0.11	0.05	0.05	1	

76% of the adults contain the transgene

F8

| | feral genotypes | | | | | | stocked genotype | | 100,000 | % |
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	10881	10881	24888	24888	14231	14231	5000	5000	110,000	10%
Adults that survive	9.89	9.89	22.63	22.63	12.94	12.94	4.55	4.55	100	
adult freq.	0.10	0.10	0.23	0.23	0.13	0.13	0.05	0.05	1	

80% of the adults contain the transgene

F9

| | feral genotypes | | | | | | stocked genotype | | 100,000 | % |
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	8993	8993	24424	24424	16583	16583	5000	5000	110,000	10%
Adults that survive	8.18	8.18	22.20	22.20	15.08	15.08	4.55	4.55	100	
adult freq.	0.08	0.08	0.22	0.22	0.15	0.15	0.05	0.05	1	

84% of the adults contain the transgene

F10

| | feral genotypes | | | | | | stocked genotype | | 100,000 | % |
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	7432	7432	23690	23690	18878	18878	5000	5000	110,000	10%
Adults that survive	6.76	6.76	21.54	21.54	17.16	17.16	4.55	4.55	100	
adult freq.	0.07	0.07	0.22	0.22	0.17	0.17	0.05	0.05	1	

86% of the adults contain the transgene

F11

| | feral genotypes | | | | | | stocked genotype | | 100,000 | % |
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	6142	6142	22765	22765	21093	21093	5000	5000	110,000	10%
Adults that survive	5.58	5.58	20.70	20.70	19.18	19.18	4.55	4.55	100	
adult freq.	0.06	0.06	0.21	0.21	0.19	0.19	0.05	0.05	1	

89% of the adults contain the transgene

F12	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	5076	5076	21711	21711	23213	23213	5000	5000	110,000	10%
Adults that survive	4.61	4.61	19.74	19.74	21.10	21.10	4.55	4.55	100	
adult freq.	0.05	0.05	0.20	0.20	0.21	0.21	0.05	0.05	1	
	91% of the adults contain the transgene									

F13	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	4195	4195	20576	20576	25229	25229	5000	5000	110,000	10%
Adults that survive	3.81	3.81	18.71	18.71	22.94	22.94	4.55	4.55	100	
adult freq.	0.04	0.04	0.19	0.19	0.23	0.23	0.05	0.05	1	
	92% of the adults contain the transgene									

F14	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	3467	3467	19399	19399	27134	27134	5000	5000	110,000	10%
Adults that survive	3.15	3.15	17.64	17.64	24.67	24.67	4.55	4.55	100	
adult freq.	0.03	0.03	0.18	0.18	0.25	0.25	0.05	0.05	1	
	94% of the adults contain the transgene									

F15	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	2865	2865	18208	18208	28926	28926	5000	5000	110,000	10%
Adults that survive	2.60	2.60	16.55	16.55	26.30	26.30	4.55	4.55	100	
adult freq.	0.03	0.03	0.17	0.17	0.26	0.26	0.05	0.05	1	
	95% of the adults contain the transgene									

F16	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	2368	2368	17027	17027	30605	30605	5000	5000	110,000	10%
Adults that survive	2.15	2.15	15.48	15.48	27.82	27.82	4.55	4.55	100	
adult freq.	0.02	0.02	0.15	0.15	0.28	0.28	0.05	0.05	1	
	96% of the adults contain the transgene									

F17	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	1957	1957	15870	15870	32173	32173	5000	5000	110,000	10%
Adults that survive	1.78	1.78	14.43	14.43	29.25	29.25	4.55	4.55	100	
adult freq.	0.02	0.02	0.14	0.14	0.29	0.29	0.05	0.05	1	
	96% of the adults contain the transgene									

F18	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	1617	1617	14751	14751	33632	33632	5000	5000	110,000	10%
Adults that survive	1.47	1.47	13.41	13.41	30.57	30.57	4.55	4.55	100	
adult freq.	0.01	0.01	0.13	0.13	0.31	0.31	0.05	0.05	1	
	97% of the adults contain the transgene									

F19	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	1337	1337	13677	13677	34986	34986	5000	5000	110,000	10%
Adults that survive	1.22	1.22	12.43	12.43	31.81	31.81	4.55	4.55	100	
adult freq.	0.01	0.01	0.12	0.12	0.32	0.32	0.05	0.05	1	
	98% of the adults contain the transgene									

F20	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	1105	1105	12655	12655	36240	36240	5000	5000	110,000	10%
Adults that survive	1.00	1.00	11.50	11.50	32.95	32.95	4.55	4.55	100	
adult freq.	0.01	0.01	0.12	0.12	0.33	0.33	0.05	0.05	1	
	98% of the adults contain the transgene									

F21

	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	913	913	11687	11687	37400	37400	5000	5000	110,000	10%
Adults that survive	0.83	0.83	10.62	10.62	34.00	34.00	4.55	4.55	100	
adult freq.	0.01	0.01	0.11	0.11	0.34	0.34	0.05	0.05	1	

99% of the adults contain the transgene

F22

	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	755	755	10775	10775	38470	38470	5000	5000	110,000	10%
Adults that survive	0.69	0.69	9.80	9.80	34.97	34.97	4.55	4.55	100	
adult freq.	0.01	0.01	0.10	0.10	0.35	0.35	0.05	0.05	1	

99% of the adults contain the transgene

F23

	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	624	624	9921	9921	39456	39456	5000	5000	110,000	10%
Adults that survive	0.57	0.57	9.02	9.02	35.87	35.87	4.55	4.55	100	
adult freq.	0.01	0.01	0.09	0.09	0.36	0.36	0.05	0.05	1	

99% of the adults contain the transgene

F24

	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	515	515	9122	9122	40363	40363	5000	5000	110,000	10%
Adults that survive	0.47	0.47	8.29	8.29	36.69	36.69	4.55	4.55	100	
adult freq.	0.00	0.00	0.08	0.08	0.37	0.37	0.05	0.05	1	

99% of the adults contain the transgene

F25

	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	426	426	8378	8378	41196	41196	5000	5000	110,000	10%
Adults that survive	0.39	0.39	7.62	7.62	37.45	37.45	4.55	4.55	100	
adult freq.	0.00	0.00	0.08	0.08	0.37	0.37	0.05	0.05	1	

99% of the adults contain the transgene

F26

	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	352	352	7687	7687	41961	41961	5000	5000	110,000	10%
Adults that survive	0.32	0.32	6.99	6.99	38.15	38.15	4.55	4.55	100	
adult freq.	0.00	0.00	0.07	0.07	0.38	0.38	0.05	0.05	1	

99% of the adults contain the transgene

F27

	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	291	291	7046	7046	42663	42663	5000	5000	110,000	10%
Adults that survive	0.26	0.26	6.41	6.41	38.78	38.78	4.55	4.55	100	
adult freq.	0.00	0.00	0.06	0.06	0.39	0.39	0.05	0.05	1	

99% of the adults contain the transgene

F28

	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	240	240	6453	6453	43306	43306	5000	5000	110,000	10%
Adults that survive	0.22	0.22	5.87	5.87	39.37	39.37	4.55	4.55	100	
adult freq.	0.00	0.00	0.06	0.06	0.39	0.39	0.05	0.05	1	

100% of the adults contain the transgene

F29

	feral genotypes						stocked genotype		100,000	%
	XX	XY	XX^	XY^	XX^^	XY^^	XX^^	XY^^	tot. juvs	stocked
100000 juvs surviving	199	199	5907	5907	43895	43895	5000	5000	110,000	10%
Adults that survive	0.18	0.18	5.37	5.37	39.90	39.90	4.55	4.55	100	
adult freq.	0.00	0.00	0.05	0.05	0.40	0.40	0.05	0.05	1	

100% of the adults contain the transgene