Problems and prospects of hormone, chromosome and gene manipulations in fish

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Hormonal induction of sex reversal is possible in 50 species. Dietary administration may be avoided by adopting immersion technique. Neuropeptide Y, aromatase inhibitor and temperature are now used to induce sex reversal.

Embryonic events namely, insemination, second polar body extrusion and suppression of the first mitotic cleavage are manipulable to render 37 different types of ploidy induction possible. In combination with cryopreservation, androgenesis restores species. Unconventional pathways of gametogenesis in fertile triploids are described. Any method that increases heterozygosity enhances viability of tetraploids.

More than 40 fish growth hormone cDNA and genomic sequences are isolated, characterized and used for construction of 'all-fish' gene cassettes for transformation. Non-invasive methods of sample collection by drawing blood and clipping fins are newly used to confirm the stable integration. Mosaic integration is an ubiquitous feature of transgenic fish. An integrated transgene may be excluded during successive cell cycles.

THIS contribution is based on our previous publications on hormonal induction of sex reversal, ploidy induction and sex control, and transgenesis in fish[1–5]. During the last two decades, numerous publications reported new methods/protocols for these manipulations in more and more species; for instance, the number of protocols available for successful hormonal induction of sex reversal increased between 1983 and 1995 from 15 species using one of the 14 steroids to 47 species using one of the 31 steroids[4]. Thorgaard[6] summarized the procedures developed for gynogenesis, triploidy and tetraploidy in a few ornamental and food-fishes; Pandian and Koteeswaran[5] reviewed the procedures developed for 37 different types of ploidy induction and indicated the amenability of a single fish species to 8–12 different types of ploidy induction. Culp *et al.*[7], Devlin *et al.*[8] and Sheela *et al.*[9] reported that their transgenics displayed dramatic growth, which was 2–12 times faster than their respective controls. More seriously some of these protocols/techniques are scaled up to industrial level (e.g. Benfey *et al.*[10] and Recoubratsky *et al.*[11]) and are being effectively utilized in aquaculture farms. Therefore, it has become necessary to update and critically evaluate the protocol and techniques developed for these manipulations.

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Hormonal induction

The process of sex differentiation in teleosts is diverse and labile[12], rendering hormonal induction of sex reversal possible. In recent years, sex control through exogenous administration of steroid has assumed significance in aquaculture operations. Of the techniques available to mass produce a sterile or monosex population, the hormonal induction of sex reversal is the most widely practised[4]. For example, if a population of only males is desired, an androgen is administered through diet or water to a population of young fish; the genetic females are induced to develop as functional males but the genotypic males are apparently not affected and therefore develop as normal, functional males. However, the technique suffers from the following problems: (i) inconsistency in dose and duration of treatment, (ii) residues of the administered hormone being carcinogenic and/or interfering with the consumers sex, (iii) need to be used every time, when desired to produce a monosex population and costs time and money, (iv) a stressful process resulting in low survival of sex reversed individuals, especially those having homogenous genotype (XX or ZZ), (v) occurrence of unexpected sex and sex ratio among the induced progenies and (vi) delayed sexual maturity and reduced fecundity.

To ensure the desired sex reversal, a chosen steroid is administered through diet or water at the optimal dose during the labile period, which corresponds with the period of initial gonadal differentiation. Most of the publications, which describe the protocol for hormonal induction of sex reversal, have therefore attempted to determine the optimum dose to induce complete sex reversal in 100% of the treated individuals and to delineate the labile period of a fish species, during which the sex reversion is possible.

Dose and treatment duration

Table 1 shows the recommended values for the dose and treatment duration for masculinization of tilapias, which vary widely; for instance, the labile period reported

by different authors ranges between 11 and 69 days for *Oreochromis mossambicus* and 25 and 59 days for *O. niloticus*; likewise, the recommended dose for 17α-methyltestosterone (MT) ranges from 5 to 1000 mg/kg diet for *O. mossambicus* and 5 to 60 mg/kg diet for *O. niloticus*. Besides, many have secured only 47–98%, despite using high dose of a very potent synthetic androgen like MT. The lowest optimal dose and treatment duration for 100% masculinization of tilapia population in the laboratory are 5 mg MT/kg diet and from 9 to 20 days after hatching[13]. Exploring the causes for such inconsistent values reported by previous workers, Pandian and his associates identified the following as partly responsible: (i) storage conditions for hormone and diet, (ii) purity and solubility of steroid and (iii) modifying effect of temperature.

Comparing conditions for hormonal sex reversal, Varadaraj *et al.*[14] recommended a procedure, which was shown to retain the maximum potency of the hormone; the procedure involves storage of feed in dark at room temperature and addition of steroid, which was previously stored in a dark space at 4°C. Level of purity maintained in the manufacturing process of the steroid used by different authors differs from company to company and can considerably modify its sex reversing potency (Figure 1; Pandian and Varadaraj[15]). Solubility of 17α-ethylnyltestosterone (ET) is maximum in the solvent dimethyl-sulphoxide (DMSO); hence 50 mg steroid (dissolved in DMSO)/kg diet induces 100% sex reversal in *O. mossambicus*, whereas an equal dose of ET dissolved in acetone or ethanol induces only 54–60% males[16]. However, the smell of DMSO, unless totally evaporated, may not sustain continued feeding. Varadaraj *et al.*[14] have also tested the effect of temperatures from 22 to 38°C, when *O. mossambicus* was administered at the optimum dose of MT through diet during the labile period; among the tested temperatures, 27°C is the optimum, as it allows 100% sex reversal into male (Table 2). Analysis of variance indicates that masculinization is more dependent on temperature than MT dose; interaction between MT dose and temperature also significantly influences masculinization. Working on the

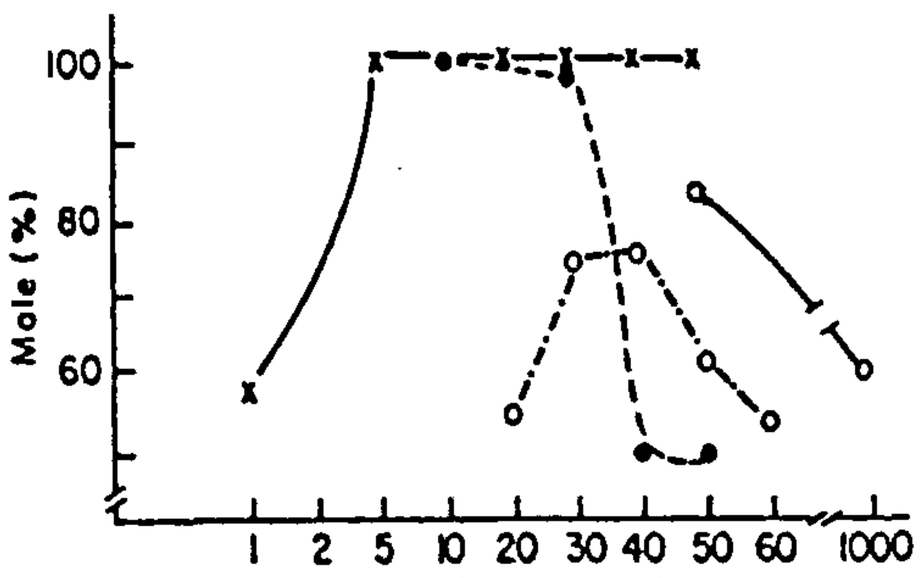


Figure 1. Variations in the sex-reversing potency of 17α-methyltestosterone administered through diet by different authors: o – – – o, Clemens and Inslee; o – · – · – o, Nakamura; o —— o, Das *et al.*; × —— ×, Pandian and Varadaraj (all from Pandian and Varadaraj[15]).

Table 1. Recommended dose for dietary administration of 17α-methyltestosterone (MT) and treatment duration to induce masculinization in tilapia species (from Pandian)[1]

Species	Dose (mg/kg/diet)	Duration (day)	Masculinization (%)
O. mossambicus	10	69	100
	30	69	100
	50	19	100
	50	30	100
	60	42	100
	5	11	100
	20	69	95
	40	69	88
	50	69	88
	1,000	19	1.5
	1,000	44	0
	30	14–28	69–98
	50	42	81–85
	30	30	47
	30	30	79
	500*		**
O. aureus	60	39	100
O. aureus	30–60	18	98–100
	30	32	100
	60	21–28	99–100
O. niloticus	5	42	100
	50	30	100
	30–60	25–59	100
T. xilli	50	45	100

*Administration through water: μg/l; **High ratio of ♀♀.

Table 2. Effect of temperature on the sex-reversing potency of 17α-methyltestosterone (MT) fed to *Oreochromis mossambicus*. Nine-day-old fry were fed 5 or 10 mg MT/kg diet for 11 days (from Varadaraj *et al.*)[14]

Temperature (°C)	17α-MT dose (mg/kg diet)	Sex ratio (♀ : ♂$_+$: ♂)
22	0	0.42 : 0.00 : 0.58
22	5	0.39 : 0.07 : 0.54
22	10	0.36 : 0.06 : 0.58
25	0	0.45 : 0.00 : 0.55
25	5	0.30 : 0.09 : 0.61
25	10	0.23 : 0.12 : 0.65
27	0	0.41 : 0.00 : 0.59
27	5	0.00 : 0.00 : 1.00
27	10	0.00 : 0.00 : 1.00
33	0	0.46 : 0.00 : 0.54
33	5	0.00 : 0.31 : 0.69
33	10	0.02 : 0.35 : 0.63
38	0	0.44 : 0.00 : 0.56
38	5	0.61 : 0.39 : 0.00
38	10	0.73 : 0.27 : 0.00

modifying effect of temperature on sex reversing potency of MT in *O. niloticus*, Baroiller *et al.*[17] have also come to the same conclusion.

With reference to labile period, Pandian and Sheela[4] have broadly recognized two major groups among teleosts; the first group includes species, in which differentiation occurs just following hatching and lasts for a short period of 10–49 days. Many ornamental fishes like the Poecilids, Anabantids and others like the Cichlids, belonged to the first group. The second group comprises foodfishes like the Salmonids and Cyprinids, in which differentiation occurs during the late juvenile stage and lasts for a period of more than 150 days (see Yamazaki[18]; Shelton and Jensen[19]). As a consequence of this difference in the labile period, the intensity of treatment to ensure 100% sex reversal is also correspondingly increased (Figure 2). The fact that the labile period is short and terminated long before the Cichlids attain harvestable body weight, confers two advantages; firstly, the cost of hormone treatment is less and secondly, the residual level of administered steroids may be so little that it may not evoke concern from consumer. Conversely, the need for a longer and intensive treatment in foodfishes like the Cyprinids may attract consumer resistance, as the fishes have already attained table size during the treatment period itself.

Mode of administration

Secondly, the orally administered steroid apparently suffers a greater loss; for instance, an interperitoneal administration may require just a tenth of that required through diet[20]. Thirdly, more than 99% of the hormone, administered through diet, is released into water in less than 24 h (ref. 21) and can be an important source of environmental pollution. Administration of hormone through immersion appears to be a better alternative, especially for foodfishes like the Salmonids and Cyprinids. For the treatment it is restricted to a short period from pre-embryonic to post-embryonic stage and can be confined to laboratory level operation, involving less water, which can easily be treated. Table 3 provides selected representative foodfishes, which are successfully sex reversed through immersion technique. Notably, protocols for hormonal induction of sex reversal by

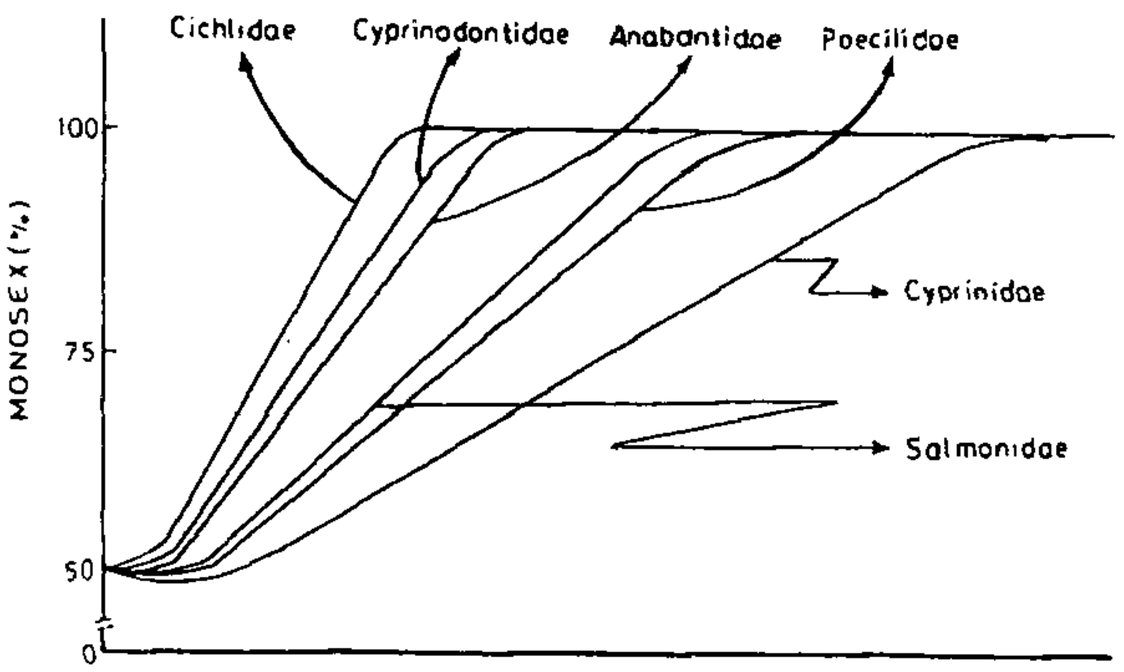


Figure 2. Suggested trends of hormonal sex reversal as a function of treatment intensity for different teleost families (from Pandian and Sheela[4]).

Table 3. Hormonal induction of sex reversal through immersion in selected teleosts

Species	Optimized protocol	Ref.
Salmonidae		
Oncorhynchus masou	0.5–5 µg E/l for 18 D; 5 DAH	Nakamura[116]
O. tshawytscha	200 µg MT/l; two 2 hr immersion; 7 & 14 DAH	Baker *et al.*[117]
O. rhodrus	400 µg E/l for 2 hr; 17 DAH	Nakamura[118]
O. kisutch	400 µg E/l; two 2 hr immersion; 1 & 8 DAH	Piferrer *et al.*[119]
O. mykiss	400 µg MT/l for 2 hr; 7 DAH	Fiest *et al.*[120]
Cichlidae		
Oreochromis mossambicus	5 mg ET/l; 10–20 DAH	Pandian and Varadaraj[15]
O. niloticus (♀) × *O. mossambicus* (♂)	320–480 µg E/l for 2–4 hr; after hatching	Koteeswaran and Pandian[121]
Cyprinidae		
Misgurnus mizolepis	200 µg E/l; 1–21 DAH	Kim *et al.*[122]
Paralichthydae		
Paralichthys olivaceus	1–10 µg E/l; 31–59 DAH	Tabata[123]
Siluridae		
Clarias gariepinus	10–100 µg MT*/l; 14–42 DAH	Hurk *et al.*[124]

DAH, Days after hatching; E, Estradiol 17β; MT, 17α-Methyltestosterone; ET, Ethynyltestosterone; *, Paradoxical feminization.

immersion technique for the Indian major carps are not yet available and urgently required.

Besides, other techniques have also been developed to ensure the production of monosex population. For instance, neuropeptide Y (NPY), which is known to control the release of gonadotropic hormone-2 in gonochoristic teleosts, has been shown to successfully reverse the sex of the protogynous bluehead wrasse, *Thalassoma bifasciatum*[22]. Likewise the administration of aromatase inhibitor may reverse the sex of female and result in the production of monosex population; for instance, the intraperitoneal implantation of fedrozole at the dose of (100 mg/kg fish) inhibits early oocyte development in female coho salmon *Oncorhynchus kisutch* (Hong and Donaldson[23]). It is likely more such new substances are identified and made available for large scale sex reversal in aquaculture farms.

The second alternative method is the application of temperature as an inductor of sex reversal. A series of recent publications by Strussmann *et al.*[24–27] and others[28] has clearly documented the thermal lability of sex differentiation in a number of teleosts. For instance, Strussmann *et al.*[24] obtained 100% females in the atherinid fish, *Odontesthes bonariensis*, when juveniles were transferred to colder temperatures of 13–19°C and 100% males, when they were reared at warmer temperature of 29°C. Appropriate protocols for the thermal induction of sex reversal in more and more teleosts are being reported[29]. Whereas hormonal induction of sex reversal requires skilled labour and costly chemicals and may attract consumer resistance, thermal manipulation to reverse the sex may prove to be the cheapest and easiest technique and can easily be practised for mass production by unskilled farmers or by using simple equipment for induction.

Negative effects

Reviewing the relevant literature on hormonal induction of sex reversal in fish, Pandian and Sheela[4] pointed out that hormonally sex reversed fish may suffer higher mortality, slower growth and reduced fecundity. It is not yet known whether such fishes, whose sex was reversed through thermal induction, would also suffer higher mortality, slower growth and less fecundity. It is also recorded that not all the individuals treated at optimal hormonal conditions were amenable for sex reversal[30]. It is not unusual for authors to report sex reversal in less than 100 treated individuals under almost optimal conditions. A possible solution to minimize such negative responses of treated fish appears to be presented by Melard[31]. He intended to generate a broodstock for production of all-male progenies in *O. aureus*, but obtained most unexpectedly a certain percentage (≈ 32%) of females among the F_1 progenies sired by hormonally sex-reversed pseudofemales, crossed with normal males (Figure 3). However, he repeated the processes of hormonal induction and progeny testing and found far less (> 2%) females among the F_2 progenies. His study suggests the progressive elimination of autosomal genes responsible for modifying the expected sex ratio among the progenies through successive generations.

Besides, there are also other instances, in which the amenability to sex reversal is restricted to one sex; for instance, the Cichlid, *Cichlosoma nigrofasciatum* was amenable for 100% feminization but not for masculinization[30]. Thirdly, hormonal induction of sex reversal also results in sterility, intersexuality and hermaphroditism among the treated individuals; even in a sex-reversed functional individual, 'the left overs' of the structures/organs related to the genotype are reported to be present. For instance, not only functional ovaries were present in the masculinized *Clarias lazera* but also self-fertilization was recorded to occur[32]. Lastly, there is also a need to eliminate the cost of time and money required for the production of monosex population, when desired. These problems have led to the production of broodstock, from which it may be possible to consistently produce a monosex population.

Broodstock development

Figure 4 illustrates representative endocrine protocols for establishing broodstocks for the production of all-male and female progenies in oviparous and viviparous fishes. Two points must be indicated here: the alternate technique

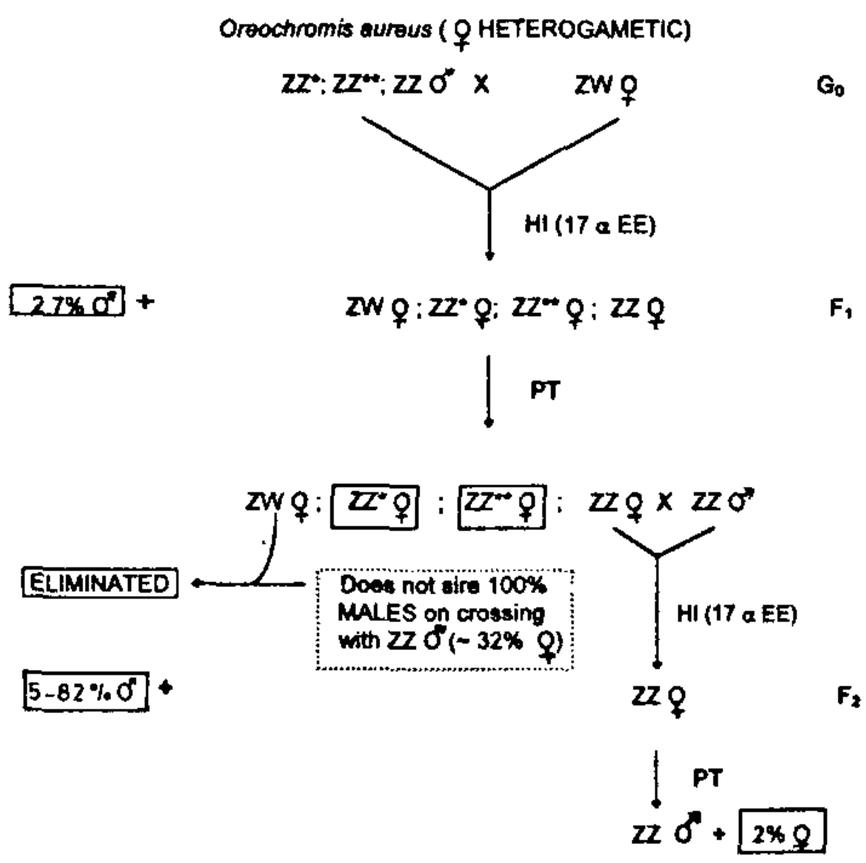


Figure 3. Schematic representation of all-male production in *Oreochromis aureus* through sex reversal and progeny testing (source: Melard[31]). HI, Hormonal induction of feminization using 17α-ethynylestradiol; PT, Progeny testing; ZZ*, Male with poly factorial sex determination; ZZ**, Male without polyfactorial sex determination (?), ZZ♀, Pseudo females.

of ploidy induction for sex reversal cannot be adopted in viviparous species, hormonal induction may have to be repeated through F_1 and F_2 progenies, when the production of YY males or YY females are desired, respectively. In oviparous species, F_1 progenies require hormonal induction and progeny testing to identify XX male, which may serve as a broodstock (when crossed with normal male) for the production of all female F_2 progenies. In both oviparous and viviparous species, production of the YY male requires hormone treatment of F_1 progenies and progeny testing to identify the XY female among the F_1 progenies and to distinguish homogametic male from the heterogametic male among the F_2 progenies. However, it has been repeatedly indicated that a cross between XX female and YY male has resulted in the production of a certain percentage of females[1].

Chromosome manipulation

In oviparous teleosts, early embryonic events such as insemination, second polar body extrusion, suppression of first cell division are manipulable (Figure 5) and render 37 different types of ploidy induction possible;

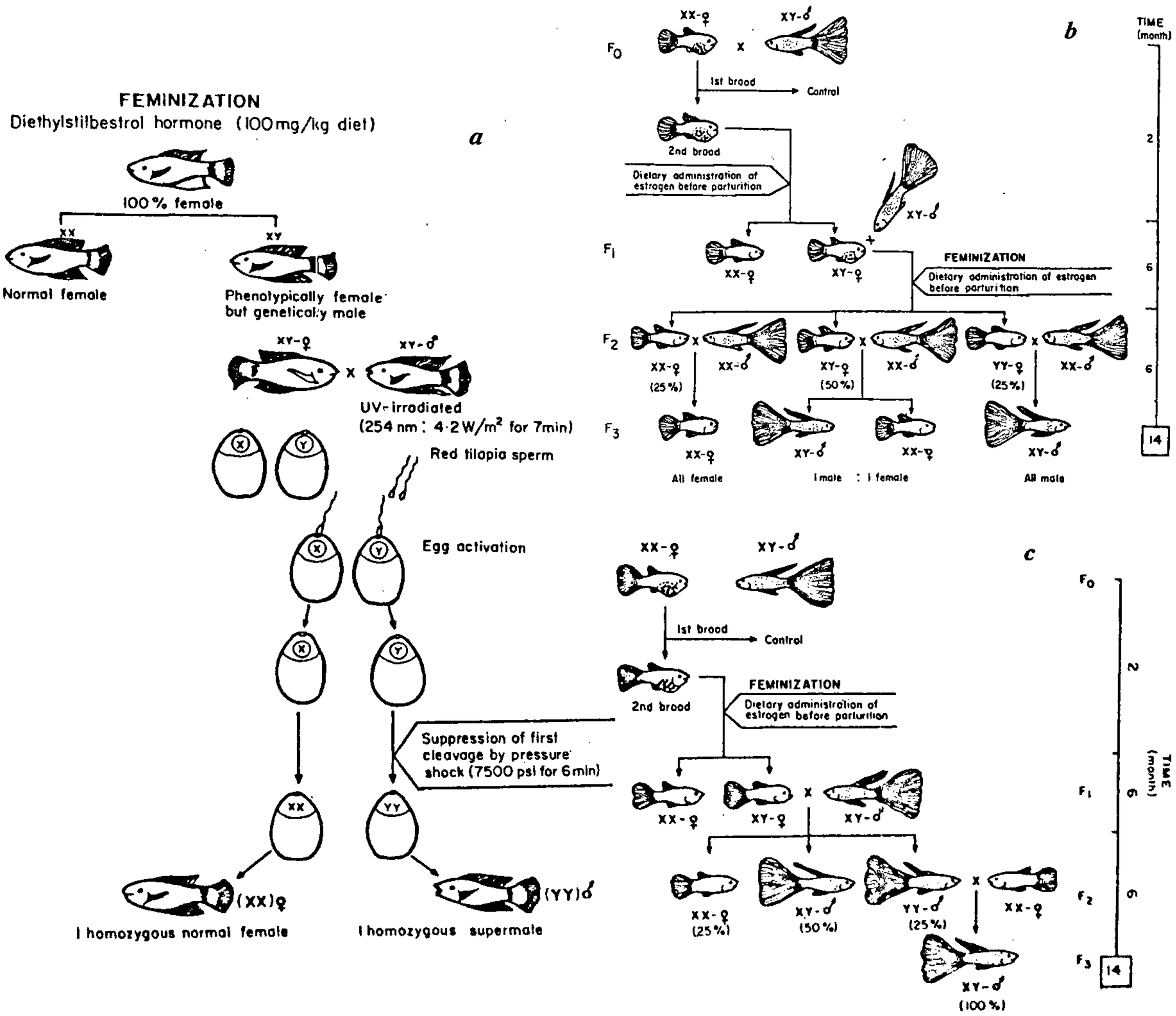


Figure 4. *a*, Procedure for generating supermale *Oreochromis mossambicus* by integrating hormonal sex reversal and gynogenesis (from Pandian[1]); *b*, Procedure for generating all-female or all-male guppy *Poecilia reticulata* by hormonal induction of sex reversal (from Pandian[1]) and *c*, Procedure for generating YY females in guppy *P. reticulata* by hormonal induction of sex reversal (from Pandian[1]).

such ploidy inductions can also result in the production of all-male, all-female or all-sterile populations[5]. The most striking features of these 37 viable combinations of genomes are: (i) the ability of a haploid vertebrate to survive, (ii) the ability to tolerate multiplication of its chromosome set to a level of heptaploidy, unusual for vertebrates, (iii) the ability to tolerate genomic contributions from a male or female parent alone; for instance, haploids, gynogens and androgens may inherit only maternal or paternal genomes, (iv) the ability to tolerate an unequal genomic contribution from male and female parents of the same species; for instance, maternal triploids tolerate genomic contributions from female and male parents at the ratio of 2 : 1, but the induced heptaploid loach tolerates the genome contributed at 6 : 1 ratio, (v) the ability to tolerate unequal or equal genomic contributions from parents belonging to different species and (vi) the ability of a species to be amenable for more than 8–10 different types of ploidy induction.

Listing selected types of ploidy inducible in fish, Table 4 identifies problems and suggests solutions to minimize or solve these problems. Gynogenetic or androgenetic haploids may be useful in studies on control of polymorphic genetic loci. Despite considerable effort, Varadaraj[33] alone has been successful in producing viable gynogenetic haploid tilapia, as they suffer heavily from haploid syndrome. Recently, the natural occurrence of monoploid (haploid male) and polyploids (triploid, tetraploid) in a catfish (*Heteropneustes fossilis*) has been reported[34]. Use of hybrid egg or sperm and heterologous insemination are the suggested methods for improving chances for successful induction of haploids.

Manipulable events

1. Normal fertilization
2. Use of genome-inactivated sperm
3. Use of genome-inactivated egg
4. Use of sperm from other species
5. Retention of polar body
6. Suppression of first mitotic division

Combinations of manipulated events

1	:	Diploidy
2, 3 or 4	:	Haploidy
2 & 5, 4 & 5	:	Meiotic gynogen
2 & 6, 4 & 6	:	Mitotic gynogen
3 & 6	:	Androgenic diploid
1 & 5	:	Triploidy
1 & 6	:	Tetraploidy
1 & 5	:	Pentaploidy*
1,2/4 & 5	:	Hexaploidy**
1 & 5	:	Heptaploidy**

*Using egg from 4*n*♀; **Using unreduced egg from triploid ♀.

Figure 5. Embryonic events that can be manipulated to induce ploidy in fish. The combinations of manipulated events that lead to the production of haploidy to heptaploidy are indicated (from Pandian and Koteeswaran[5]).

Gynogenesis

It is a developmental process facilitating the inheritance of maternal genetic material alone. It involves two steps: (i) elimination of paternal chromosomes, i.e. activation of egg by irradiated homologous sperm, or intact or irradiated heterologous sperm and (ii) restoration of diploidy by a shock to retain the second polar body (meiogynogenesis) or to suppress the first mitotic division (mitogynogenesis). Retention of the second polar body permits effective duplication of the genome without interfering with crossing over events, but guarantees some levels of heterozygosity[35]. Therefore, the products of meiotic and mitotic gynogenesis are heterozygous and homozygous gynogens, respectively. Consequently, meiotic gynogenesis can produce isogenic lines of fish within a few gynogenetic generations, but not homozygous (clonal) lines of fish[36]; homozygous lines of fish can be established by mitotic gynogenesis in two successive generations[37]. Research in gynogenesis may thus help to (i) generate all female progenies, (ii) establish stable isogenic (by crossing inbred lines) and homozygous lines useful for gene mapping, standardization of bioassays in immunological and endocrinological studies, (iii) understand the genetic regulation of development and construction of linkage map[38] and (iv) increase production, at least in some species[29].

Suitable protocols have been developed to successfully induce mitogynogens and meiogynogens in about 15 and 30 species respectively[5]. For it is technically more difficult to suppress the first cleavage than to retain the second polar body; consequently, induction of mitogynogenesis leads to significantly higher mortality (80%) than that of meiogynogenesis (30%). Application of a shock and the consequent mortality may be avoided, when a tetraploid female is crossed with a diploid male[39]. In general, higher survival is ensured, when pressure or cold shock is chosen[5]. The use of irradiated heterologous sperm, for reasons not yet known, increased the viability of gynogens. A couple of recent publications[2,40] indicates that generation of F_2 mitogynogens may considerably improve viability and fertility (Figure 6). Inducing mitotic gynogenesis, successive generations of *O. mossambicus* clones were generated; expectedly, survival of G_0 mitogynogens was low (22%); however, it increased to 52 and 64% in F_1 and F_2 progenies. Likewise, the fertility of hybrid (*Carassius auratus gibelio* ♀ × *Cyprinus carpio* ♂) gynogen increased from 22% in G_0 to 97% in F_2. These experiments clearly indicate a progressive elimination of deleterious genes (responsible for mortality and infertility) from the population[31].

Androgenesis

It is a developmental process facilitating the inheritance of paternal genetic material. It obligately involves inactivation of egg genomes and dispermic or monospermic activation by haploid gametes. It may prove useful for the production of (i) viable (YY) supermales in male heterogametic species, (ii) inbred isogenic lines and (iii) conservation of genotypes. However, it results in very low survival and high frequency of deformity. It has been difficult to totally eliminate the contamination of maternal DNA. The technique of using UV irradiation to completely eliminate egg genome has to be considerably refined. Maximal elimination of egg genome by UV irradiation, induction of androgenesis using $2n$ sperm of a tetraploid, or a hybrid tetraploid, facilitation of dispermy and activation by cryopreserved or heterologous sperm are landmark events in the history of androgenesis. In combination with cryopreservation of sperm, androgenesis may prove to be the best method for conservation of genome.

The techniques thus far suggested to improve the yield and survival of androgens may be as below: (i) Genetic nature of the sperm and quality of eggs are important in securing a higher yield of androgens[41], (ii) insemination by diploid sperm significantly improves the yield (43%) and viability of androgens[41] but reduces homozygosity by activation of genetically inactivated eggs of *Acipenser baeri* with *A. ruthenus* sperm or dispermic activation of genetically inactivated hybrid (*Cyprinus carpio* ♀ × *C. auratus gibelio* ♂) eggs[42].

Considering immense potential for the production of new strains of economically important species, there is a need to develop new techniques for the conservation of wild strains of fish. Because of the telolecithal nature of fish eggs, cryopreservation of embryos and eggs remains unsuccessful until today but the cryopreservation of sperm has been successful[43]. Being amenable to cryopreservation and induction of androgenesis, fishes are uniquely advantageous, as these techniques can be used to restore a strain or species from the cryopreserved sperm (Figure 7). Indeed, cryopreserved sperm may become the 'gene banks' for the wild strains and species that are to be conserved for future use.

Triploidy

The ease with which the second polar body can be retained by a shock has led to the production of triploids in more than 35 species during the last 30 years[5]. Yield and survival of triploids decrease with the kind of inductors used in the following order: pressure < cold < heat shock. As many as 11 different types of triploids are inducible. Bred triploids have been produced by crossing tetraploid females with diploid males. To ensure faster growth of the desired sex, monosex triploids have also been produced combining hormonal induction of sex reversal and ploidy induction.

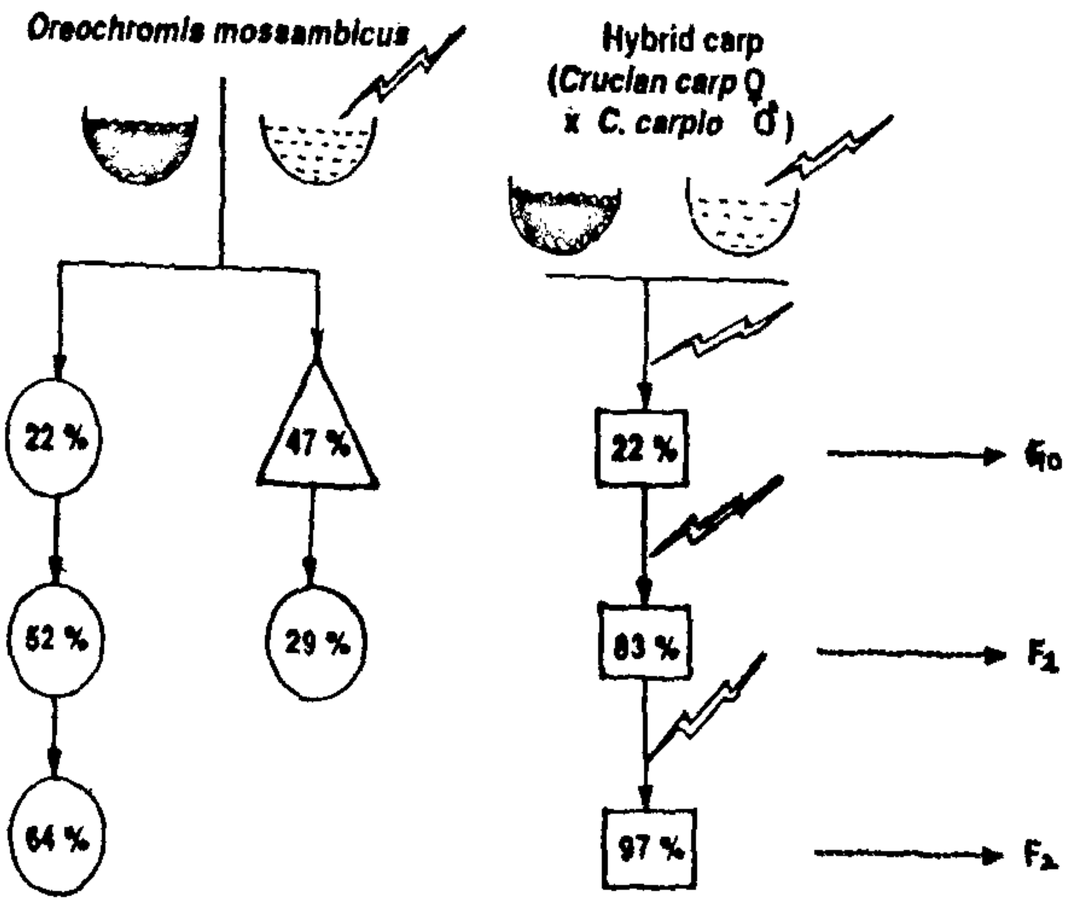


Figure 6. Survival and fertility of gynogenetic progenies of tilapia and carp (from Pandian and Koteeswaran[5]). O, Induction of mitotic gynogenesis; Δ, Induction of meiotic gynogenesis; □, activation of hybrid diploid egg; ⟋, UV irradiation of sperm.

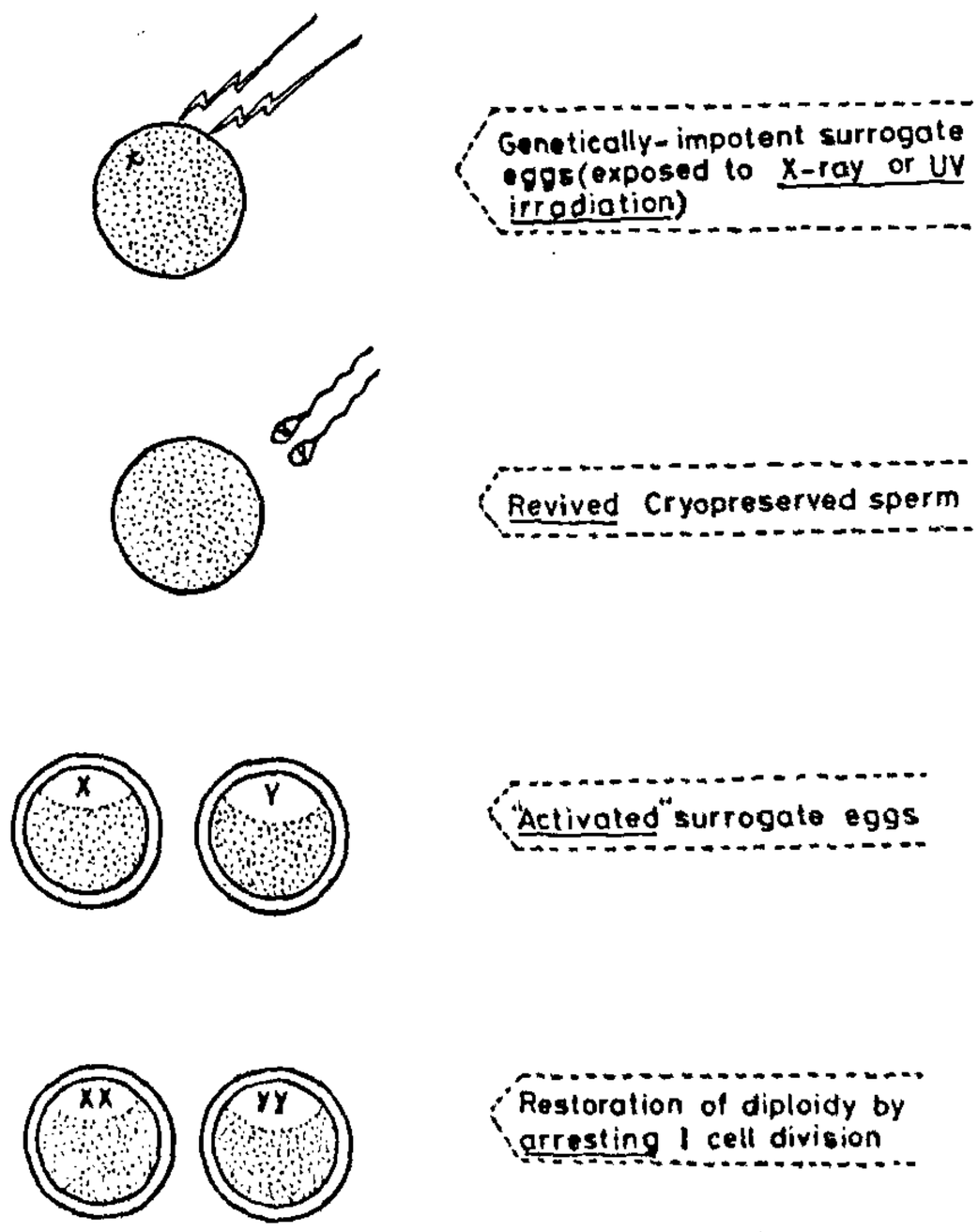
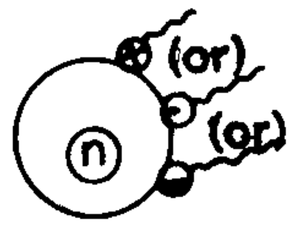
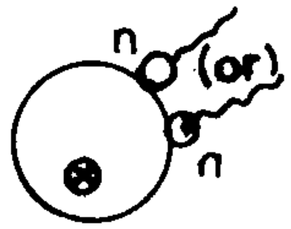
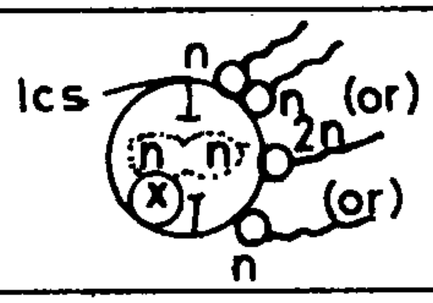


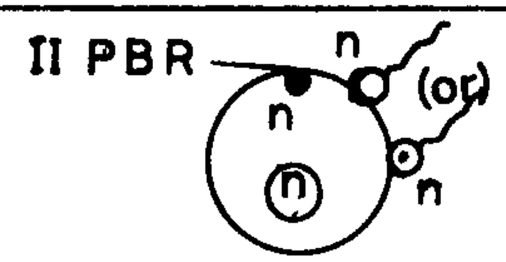
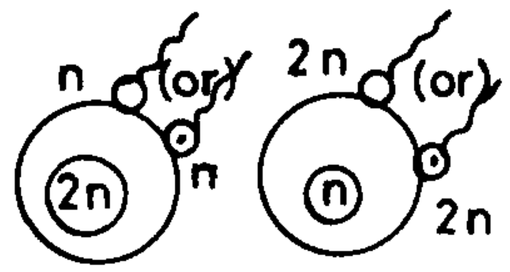
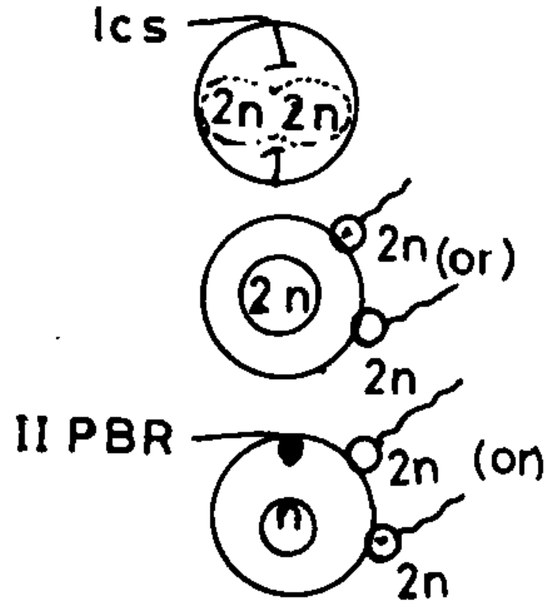
Figure 7. Restoration of endangered fish species by induction of androgenesis using cryopreserved sperm.

Table 4 . Kinds of ploidy inducible in fish : problems and solutions (♂ = Homologous (non-irradiated); ♂ = Homologous (irradiated); ♂♂ = Dispermy; ♂ = Heterologous (non-irradiated); ♂ = Heterologous (irradiated); ⊗ = Egg DNA inactivation; II PBR = II polarbody retention; I Cs = I cleavage suppression)

Ploidy Type	Protocol(s) for induction	Diagramatic representation	Problems	Solutions
HAPLOIDY *Gynogenesis*	inactivation of ♂ gamete or heterologous insemination using irradiated / non-irradiated sperm		>1-2% survival; expression of deleterious genes; very high frequency of deformity.	Use of hybrid egg (n); use of heterologous sperm;
Androgenesis	inactivation of ♀ gamete and activation with homologous heterologous sperm		>1% survival at hatching; none survives beyond hatching; very high frequency of deformity; expression deleterious genes.	i)use of hybrid egg; ii)use of heterologous eggs.
DIPLOIDY *Gynogenesis* a) meiotic	egg activation by homologous/ heterologous insemination (irradiated/non-irradiated) and II PBR.		Good survival and yield but reproductive ability low; occurrence of males among gynogens; slow growth	F_3 meiogynogens suffer less mortality
b) mitotic	egg activation and suppression of I cleavage		very low survival; highfrequency of deformity; low growth, GSI & fertility.	i)use of hybrid (2n)/tetraploid ♀ eggs (2n) ii)use of F_3 mito-gynogens.
Androgenesis	Inactivation of ♀ garhete & activation with homologous/ heterologous sperm & I cleavage suppression		Very low survival; high frequency of deformity; low GSI & fertility; maternal DNA contamination	i)use of heterologous eggs ii)sperm from outbred sources iii)sperm from tetraploid iv)use of UV for egg DNA denaturation.

Contd.

Table 1. *Contd.*

Ploidy Type	Protocol(s) for induction	Diagramatic representation	Problems	Solutions
TRIPLOIDY	Supression of II PB after homologous or heterologous insemination		Good survival and yield but different protocols for same species; low growth; in few cases, 100 % 3n is not possible to induce.	i)use of similar rearing & incubation temperatures for a given species ii) use of superovulated eggs iii)growth studies for a 3n species under solitary & communal rearing conditions iv)use of eggs & sperm from single ♀ & ♂
	Eggs from 4n♀ fertilized by 2 n ♂ & vice versa		very low survival; high frequency of deformity; slow growth.	use of F_3 tetraploids for mass producing triploids
TETRAPLOIDY	Suppressing I cleavage; using egg & sperm from tetraploid; using sperm from 4n & II PBR		Very low survival ; high frequency of deformity; 2n-4n mosaic; low growth & GSI.	i)use of F_3 tetraploids ii)use of hybrid egg (2n) & sperm (2n) iii) heterologous insemination

Retaining the second polar body, virtually all these triploids have two sets of maternal and one set of paternal chromosomes. Notable exceptions are the triploids produced by crossing tetraploid males with diploid females[44] or by the dispermic fertilization of haploid eggs after treating the spermatozoa with polyethylene glycol[45]. In these cases, the resultant paternal triploids have one set of maternal and two sets of paternal chromosomes. No comparative study has thus far been undertaken on the viability, growth and reproduction in the maternal and paternal triploids. Likewise, triploids have also not thus far induced in female-heterogametic species.

A major objective of producing triploids in aquaculture is that they are sterile and may reduce the energy costs of reproduction. The introduction of exotic sterile triploid fish to control growth of hydrophytes in man-made reservoirs and other aquatic bodies is preferred[46]. To avoid accidental introduction of undesired transgenic strains, it is recommended that after transferring the foreign gene into the fertilized eggs, the zygotes are induced to yield triploid transgenic fish[3]. In some species, hybrid triploids are more resistant to diseases than their paternal species or their diploid counterparts[47–50].

Artificial induction of hybrid triploids often results in higher survival[51], fewer developmental abnormalities and faster growth[52] than corresponding diploid hybrids. The addition of a haploid set of maternal chromosomes presumably neutralizes developmental incompatibilities occurring between the interspecific paternal genomes. In general, triploid hybridization is preferred in salmonid aquaculture, as such hybridization confers additional advantages. Salmonids like the chum salmon (*Oncorhynchus keta*) and pink salmon (*O. gorbuscha*) successfully adapt to sea water, almost immediately after commencing feeding. But others, like Atlantic salmon (*Salmo salar*), chinook salmon (*O. tshawytscha*) and coho salmon (*O. kisutch*) require freshwater for a period of few months to one year[53]. This obligate freshwater rearing is expensive. However, the meat quality of these salmons is more acceptable and fetches a 2–4 times higher price. Hence, hybrid triploids with enhanced capacities for 'smoltification' may facilitate early transfer to sea water and prevent introgression with wild salmon.

Triploidization facilitates hybridization between closely and distantly related fish species. Interspecific and intergeneric triploid hybridizations have been achieved in many species of Salmonids, Cyprinids and Cichlids[5]. Triploid hybrids involving three species, sometimes belonging to more than one genus, occur naturally. They are generally sterile; however, hybridization sometimes triggers unusual meiotic mechanism that permits hybrids to produce viable gametes. For instance, interspecific females produced from reciprocal crosses between *Oryzias latipes* and *O.l. curvinotus* were characterized by reduced fertility (see also refs 40, 54) but they did lay eggs. When fertilized by sperm from one of the parental species, these eggs developed into triploid males and females[54]. Through electrophoresis and scale transplantation studies, Sakaizumi *et al.*[55] have shown that these triploid hybrids were either *O.l. sinensis-2 curvinotus* or *O.l. 2-sinensis-curvinotus*.

Growth performance of hybrid triploid salmons has been studied on a long-term basis[56,57]. In general, triploid hybrids grow as fast as diploid hybrids; in some cases, hybrid triploids (*Salmo salar* ♀ × *S. trutta* ♂) grow faster than their diploid counterparts[58]. Habicht *et al.*[59] made a detailed study on growth rate and efficiency of triploid hybrids (coho ♀ × chinook salmon ♂) and triploid conspecific. Conspecifics convert their food at a higher efficiency than the hybrids and the triploid conspecifics grow 10–15% faster than triploid hybrids. Incidentally, triploids are sterile partially or completely; hence, it is expected that the quantum of energy, which will otherwise be channelled to reproduction, is made available for somatic growth. Figure 8 shows that at sexual maturity triploid *Betta splendens* did not grow, as fast as its diploid counterpart; in fact triploid *Pagurus major* suffered negative growth at sexual maturity. Diploid female plaice × flounder hybrid grew negatively during spawning but subsequently recovered fast to catch up the growth of its triploid counterpart. Diploid and triploid *O. gorbuscha* displayed almost comparable growth during the experimental period of more than 3 years. These examples show that partial or total sterility does not confer conspicuous advantage to triploids to channel more energy to grow faster[5].

According to the degree of gametogenesis, it is possible to recognize four types of triploids: In the first one, both males and females cannot produce gametes (e.g. *Misgurnus anguillicaudatus*[60]), in the second type males can produce spermatozoa but females cannot produce eggs (e.g. *Betta splendens*[61]), in the third one, females alone are fertile (e.g. *Carassius auratus langsdorfii*[62,63], trigenomic or digenomic hybrid triploid carps[49,64]); these triploid females, when hormonally sex-reversed, can function as normal triploid males, producing viable sperm[65,66] and in the fourth one, both males and females are fertile (e.g. hybrid triploid, *Cyprinus carpio* ♀ × *Ctenopharyngodon idella* ♂)[67]. The first and second types are common; but the third and fourth types are rare. The spermatocytes, which are present in good numbers at least in a few triploid species, fail to mature into motile, euploid spermatozoa, as spermatogenesis is progressively impaired cytogenetically[68]. In types 3 and 4 the females of some races or hybrids are fertile indicating that the oogenesis is organized and regulated through one of the suggested cytological pathways (Figure 9). For type 3, good examples are *Carassius auratus longsdorfii*[62] and *C. auratus gibelio*[66]. Clearly,

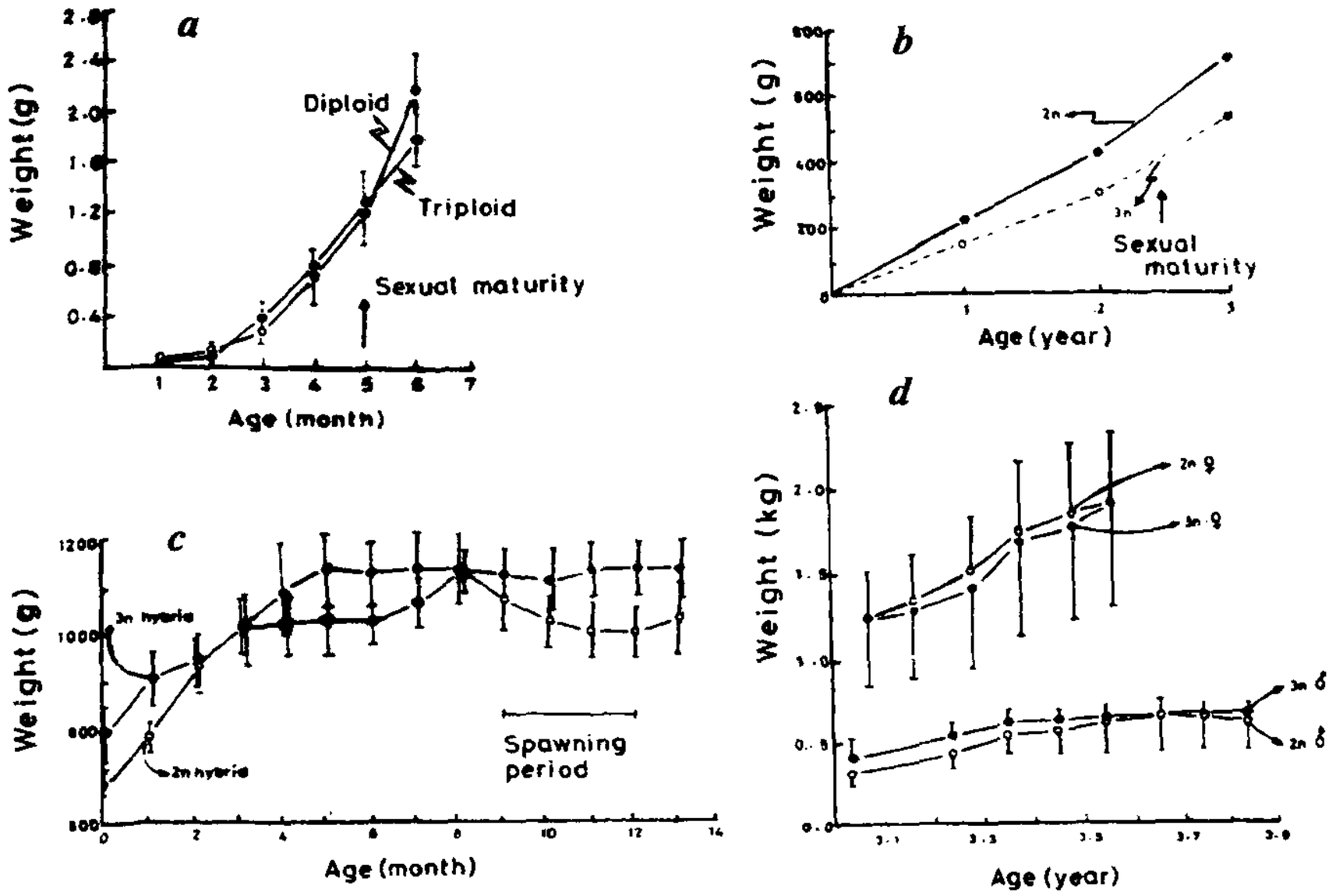


Figure 8. Growth of diploid and triploid fish. *a*, *Betta splendens*; *b*, *Pagrus major*; *c*, *Hybrid* ♀ (plaice ♀ × flounder ♂); and *d*, *Oncorhynchus gorbuscha* (source: Pandian and Koteeswaran[5]).

oogenesis is successfully completed, at least in some oocytes in these fertile triploids.

Tetraploidy

To date it has been possible to produce live, feeding tetraploids in 10 species only. In *Misgurnus anguillicaudatus*[69] and *Cobitis biwae*[39] bred tetraploids were produced by crossing 4*n* males with 4*n* females. Thorgaard *et al.*[70] successfully suppressed the first cleavage of *Oncorhynchus mykiss* to produce mitotic tetraploid. Gynogenetic tetraploid in *M. anguillicaudatus* was generated by Arai *et al.*[71] by activation of eggs of 4*n* female and retention of polar body. Induction of these tetraploids resulted in heavy mortality and poor yield and survival. Survival and growth of tetraploids have been shown to progressively improve in F_1 and F_2 tetraploid progenies[35], suggesting the progressive elimination of lethal genes during successive generations. A method of tetraploid production involving meiosis rather than mitosis may introduce a higher percentage of heterozygosity and better survival[35,57] (Table 5). A better method of increasing heterozygosity is to induce hybrid tetraploids. Kobayashi[72] generated hybrid tetraploids, by crossing fertile triploid *Carassius auratus langsdorfii* (3*n* eggs) female diploid *C. auratus auratus* male (*n* sperm). Cherfas *et al.*[40] obtained live, fertile hybrid tetraploids by crossing a hybrid (*C. carpio* ♀ × *C. auratus gibelio* ♂) male and female carp, which produced unreduced diploid gametes.

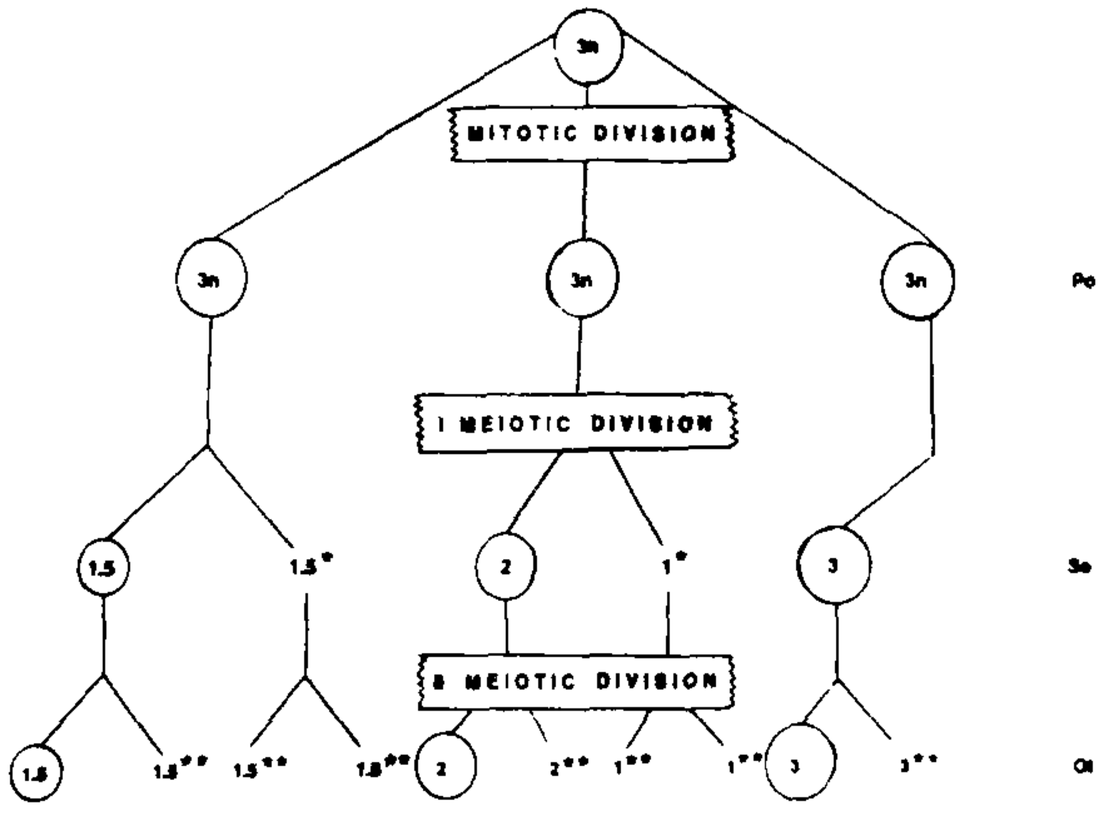


Figure 9. Oogenesis in triploids. O, oocyte/ootid; *, I polarbody; **, II polarbody; Po, primary oocyte; So, secondary oocyte; Ot, ootid/oocyte (from Pandian and Koteeswaran[5]).

The known and assumed reasons for failure to induce live tetraploid fish are: mosaicism, aneuploidy, reduced cell surface, choosing wrong cytological events and/or high homozygosity. Tetraploid mammals and birds are not viable and are known not to tolerate critical changes in the genome–cytoplasmic ratio[5]. Induction of such genomic changes is usually reported to involve considerable mosaicism[73,74]. Bidwell *et al.*[73] reported that

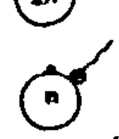
thermal induction, especially at high temperatures, resulted in a higher frequency of $2n$–$4n$ mosaicism. Most of these authors, who related tetraploid mortality to mosaicism, used flow cytometry to analyse a large number of cells from the treated fish. Using a Coulter counter, Cassani[74] estimated that in comparison to diploids, the cell number present in tetraploid grasscarp, *Ctenopharhyngodon idella* was 54% only. Such a decrease in cell number and the corresponding increase in cell volume would decrease the cumulative cell surface available for physiological reactions, which may limit or inhibit cellular metabolism.

However, generation of tetraploids is important, as it is the source for production of polyploids. There is considerable scope for successful production of tetraploids by (i) siring successive generations of mitotic tetraploids, and (ii) introduction of heterozygosity in tetraploid by involving meiosis and/or hybridization.

Gene manipulation

In recent years transgenesis has become the most widely practiced method of gene manipulation. It may be defined as the introduction of exogenous DNA into the host genome, resulting in its stable maintenance, expression and transmission[75]. The first successful gene transfer was demonstrated in mice[76]. This has triggered a series of experiments in animals including fish that are useful to man. Fish prove to be a better model for transgenesis than mammals by virtue of higher fecundity, external fertilization and development, and transparency of embryos in many species, in addition to easy manipulation of sex, ploidy and production of haploid gynogenetic and androgenetic individuals[5]. There are non-lethal, *in vivo* assays available for analysis of gene expression in fish embryos (e.g. see ref. 77), which are clearly not possible with mammalian system. Not surprisingly, there has been a continuous rain of about few hundred publications, which have been evaluated by an unprecedented number (~ 20) of reviews (e.g. refs 3, 78, 79), describing quarterly developments in this important emerging area. The land-mark progress made by these contributions has witnessed the shift in usage of (i) heterologous genes in 1985 to homologous all-fish genes in 1990 and (ii) microinjection in 1985 to electroporation by 1990; 'the egg microinjection procedures are time-consuming, laborious, species-specific and in some cases technically demanding'; the use of electroporation for gene transfer in fish appears to have removed a bottle-neck in expansion, exploration and realization of the full potential of this powerful transgenic technology[78].

Table 5. Selected crosses in the loach *Misgurnus anguillicaudatus* and progeny survival

Cross × induction	Resulting ploidy (n)	Genotype of egg/polar body	Survival (%)
$2n \times 2n$	Diploidy		40
$4n \times 4n$	Tetraploidy		36
$2n \times 4n$	Triploidy		67
$4n \times 2n$	Triploidy		18
$4n \times 2n$-HS	Pentaploidy		9
$4n \times 2n$ UV	Gyno diploidy		40
$4n \times$C UV	Gyno diploidy		31
$4n \times$C UV-CS	Gyno tetraploidy		46
$2n \times$G UV	Gyno haploidy		2
$2n \times$G UV-CS	Gyno diploidy		47

UV, UV-irradiation of the sperm; CS, cold shock; HS, heat shock; C, *Cyprinus carpio* and G, *Gnathopogon elongatus elongatus*. (From Arai *et al.*[71])

Gene constructs

Early research work on transgenic fish mostly relied on the use of gene constructs containing mammalian, avian or viral regulatory and coding sequences, as the availability of suitable alternative piscine sequences was limited[3]. Secondly, achieving good expression of heterologous genes was a problem; it was thought that due to inappropriate splicing and translation, the heterologous (e.g. mammalian) introns may not always efficiently be processed by the piscine cellular machinery[80]. The ever increasing knowledge of the structure and function of eukaryotic gene has clearly shown the need for introns, enhancer regions, boundary regions and locus control regions, besides a suitable promoter in the construction of suitable appropriate vector for the use in transgenic system. Consequently, tremendous efforts have been made during the last 10 years to isolate appropriate fish sequences; thus, more than 70 fish cDNA and genomic sequences have been isolated and characterized; (e.g. ref. 81) reports on such isolation, characterization and construction of 'all-fish' gene constructs for more and more fish species are being frequently made. Of these, only two potentially useful genes are available today to aquaculture, namely growth hormone gene and antifreeze protein gene. Most research groups from the West as well as Asian countries are attempting to develop suitable transgenic technology to achieve sustained acceleration of growth in selected foodfish. A few groups have claimed as much as 6–11 times

accelerated growth (e.g. refs 8, 82). Yet, no one has published a research document to show that such an accelerated growth has been sustained in F_1 progenies of the 'claimed' transgenic fish species. It is not clear whether such 'silence' is due to the patenting process, consumer resistance, or the problems related to expression of the transgene in F_1 progenies.

A survey of these 40 and odd growth hormone gene sequences of piscine origin clearly indicates that the constructs are available mostly for foodfish and have been developed by scientific groups from the developed countries; among developing countries, China (e.g. *Cyprinus carpio*[83]) and Israel (*Sparus auratus*[84]) have cloned GH cDNA from their indigenous fish species in attempting vector construction. India is soon to develop its own gene constructs for the Indian major carps[81] and Indian catfish[85]. With a kind of patenting and scientific intellectual property system prevailing in the international scenario, it has become a necessity for the developing countries, which depend on fisheries as an important food source, to develop 'all-fish' gene constructs for growth enhancement in fish species, that are economically important to them.

Apart from growth hormone genes, several reporter genes (Table 6) are available for transgenic studies and some of them have been used for a better understanding of integration (e.g. ref. 86), expression (e.g. refs 7, 87) and transmission (e.g. ref. 86). Few research groups have constructed gene sequences containing a growth hormone gene and a reporter gene along with other usual elements (e.g. ref. 88). Methods to detect and quantify these reporter genes are relatively simple, rapid, less cumbersome than required for growth hormone gene. Therefore, it was considered that such combination of growth hormone gene and reporter gene, and the use of such gene constructs may reduce the cost and time required for identifying putative founder transgenic fish.

Table 6. Advantages and limitations of using reporter genes in transgenesis

Method	Advantages	Limitations
CAT	Present in prokaryotes only; simple but sensitive assay protocols	Endogenous mimic activity may lead to inconclusive results
β-gal	Most versatile for different kinds of expression assays	Mimic activity leads to inconclusive results
Neo	Very sensitive	Drug unsuitable for use in live embryos; invasive to non-expressive tissues in transgenic mosaic
Tyrosinase	Sensitive, site-specific, non-invasive method; can be used in mosaic transgenic also	Use limited to only albinos
Luciferase/ GFP	Simplest but most sensitive almost without background activity	Flurometer/fluorescent microscope required

However, this consideration appears to be incorrect. For instance, Sheela *et al.*[88] electroporated a gene construct containing yellow porgy GHcDNA with β-gal reporter gene into the eggs of *Heteropneustes fossilis*. The levels of expression of GH gene, as measured by somatic growth and reporter gene, as measured by enzyme assay in the putative transgenics were 30–60% and 200–600%, respectively. Therefore, it is likely that a reporter gene like β-gal may not serve as a quantitative index for the integration and expression of a fused GH gene. Incidentally, even the plasma level of growth hormone cannot serve as a reliable quantitative index of growth, as there is no correlation between the hormonal level and growth of the transgenic Atlantic salmon[82].

Gene transfer

To transfer a selected gene construct into the zygote, microinjection was the most preferred method. The tough chorion and invisible nucleus of the fish eggs rendered the microinjection a more hazardous task; the cytoplasmic introduction of the transgene by microinjector led to its integration/persistence[89] and in some cases, even its transmission to F_1 progeny[90]. However, electroporation is presently the most preferred technique of gene transfer[87,91].

Table 7 presents a comparative account on the alternate methods of gene transfer with particular reference to survival and growth observed in the putative transgenic catfish; interestingly, zygote electroporation led to genomic integration and a greater acceleration of growth in the putative transgenics but perhaps at the cost of relatively low survival and a higher frequency of deformity among the hatchlings. More recently, Sheela *et al.*[92] reported that the observed 30–60% accelerated somatic growth in the transgenic *H. fossilis* was indeed at the cost of reduced fecundity.

Integration

Different methods have been used to confirm the integration of transgene in the putative transgenics; their advantages and limitations are briefly summarized in Table 8. Since all the methods suffer one or more limitations, it will be ideal to adopt at least two methods to confirm integration. Unfortunately, some authors have confirmed genomic integration, as confirmed by a single method (e.g. ref. 93). Among those who have adopted more than two methods to confirm genomic integration, only a few have used restriction enzymes and hybridization of the transgene to confirm not only the integration but also the rearranged form, in which it is integrated[88,94].

For want of adequate tissue to secure the minimum required DNA, many have chosen to use few indi-

Table 7. Comparison of alternative methods on gene transfer in catfish using pRSVrtGH (from Sheela[99])

Method	Treated eggs (no/min)	Survival at hatching (%)	Integration as per slot blot (%)	Type of integration (Southern)	Growth deviation of transformants at 4 months	Deformities in transformants
Micro-injection	> 1	19	24	1	++	+
Sperm-electroporation	≅ 100	11	23	1	+++	++
Electroporation	≅ 100	15	27	2	+++	+++
Sperm-incubation	< 100	36	5	1	+	+

1, Extrachromosomal; 2, Genomic.

Table 8. Advantages and limitations of methods that are most commonly used for identification of transgenes in putative transgenic animals

Method	Advantages	Limitations
Slot blot	Faster and simpler than Southern; Quantification of transgene possible	Transgene integration cannot be confirmed
Southern	Most reliable to confirm integration. Identification of rearranged forms of transgene possible	Involves a laborious protocol. Need for a relatively higher quantity of DNA limits its application for small ornamental fishes and where the tissue sample is precious
PCR	Rapid method; Requires minimum sample of DNA; Radioisotopes not needed	DNA contamination due to mis-handling results in 'illegal' amplification

viduals[86,95]. However, it is likely that some of them may be putative, while others are not. To avoid invasive method, drawing blood (e.g. ref. 82) or clipping the fin[88,96] is used as a non-invasive method to collect sample for DNA extraction from the putative transgenics. Sheela *et al.*[88] made a unique design of experiment to cull successive 7 fin clippings from the same putative transgenic *H. fossilis* over a period of 8 months to confirm the stable integration (Figure 10). Even such a non-invasive method fin clipping and analysis by Southern and other techniques may not conclusively prove the genomic integration in a putative individual. For instance, a few putative transgenic zebrafish were found to be germ-line positive but fin negative[96].

Mosaicism

Many authors have reported detailed information on the occurrence of mosaicism in a number of putative transgenic fish[9,88,97,98]. Since even extrachromosomal maintenance of transgene is known to result in germ-line transmission, germ-line transmission by itself should not be considered as a proof for stable genomic integration. Integration should be substantiated by a direct demonstration of linkage between injected sequence and the chromosomal DNA of the transgenics[96]. Moreover, the delivered DNA may remain extrachromosomal even after recombining with genomic DNA of the transgenic[96]. For this reason, it is considered important to confirm integration by the ratio of Mendelian inheritance ratio (through generations), which is the most stringent criterion for chromosomal integration. Unfortunately, only a very few authors have confirmed the integration using the Mendelian inheritance ratio as a criterion. Stuart *et al.*[96] reported the occurrence of 20% frequency of transformants among F_1 progenies; they suggested that many fin-positive founder parents were perhaps mosaic in germ cells. Observing 50% frequency of transformants among F_1 zebrafish progenies, Sheela *et al.*[9] suggested the possibility of transgene insertion into a single allele.

Although only a few authors have described it, mosaic integration appears to be an ubiquitous feature of transgenic fish. Table 9 shows that among the transgenic individuals, an individual may be positive for fin but negative for gonad, while the other may be negative for fin but positive for gonad. Secondly, it also indicates that the observed mosaicism is not common to all the tested organs that are differentiated from one or the other germinal layer, but occurs randomly, probably throughout the embryonic development. This is evidenced by the fact that in a number of fish, cellular level mosaicism has also been observed; for instance, only 20% of the germ cells in the gonad of putative transgenic was shown to be positive[99]. This would imply within a single organ like the gonad, 20% cells are transgenic, while others are not. Moreover, the number of transgene copies present is also known to vary from cell to cell, organ to organ and individual to individual (e.g. refs 100, 101). Mosaicism appears to pose a major problem to the generation and establishment of transgenic animals.

Transmission

Table 10 shows that even among the transgenic fish biologists, only a few have reported reliable information on transmission of GH gene; many publications (e.g. refs 91, 102, 103) remain silent regarding transmission. Expectedly, a long experimental period required for this kind of study has limited publication on this aspect. The results reported by most of the authors for the

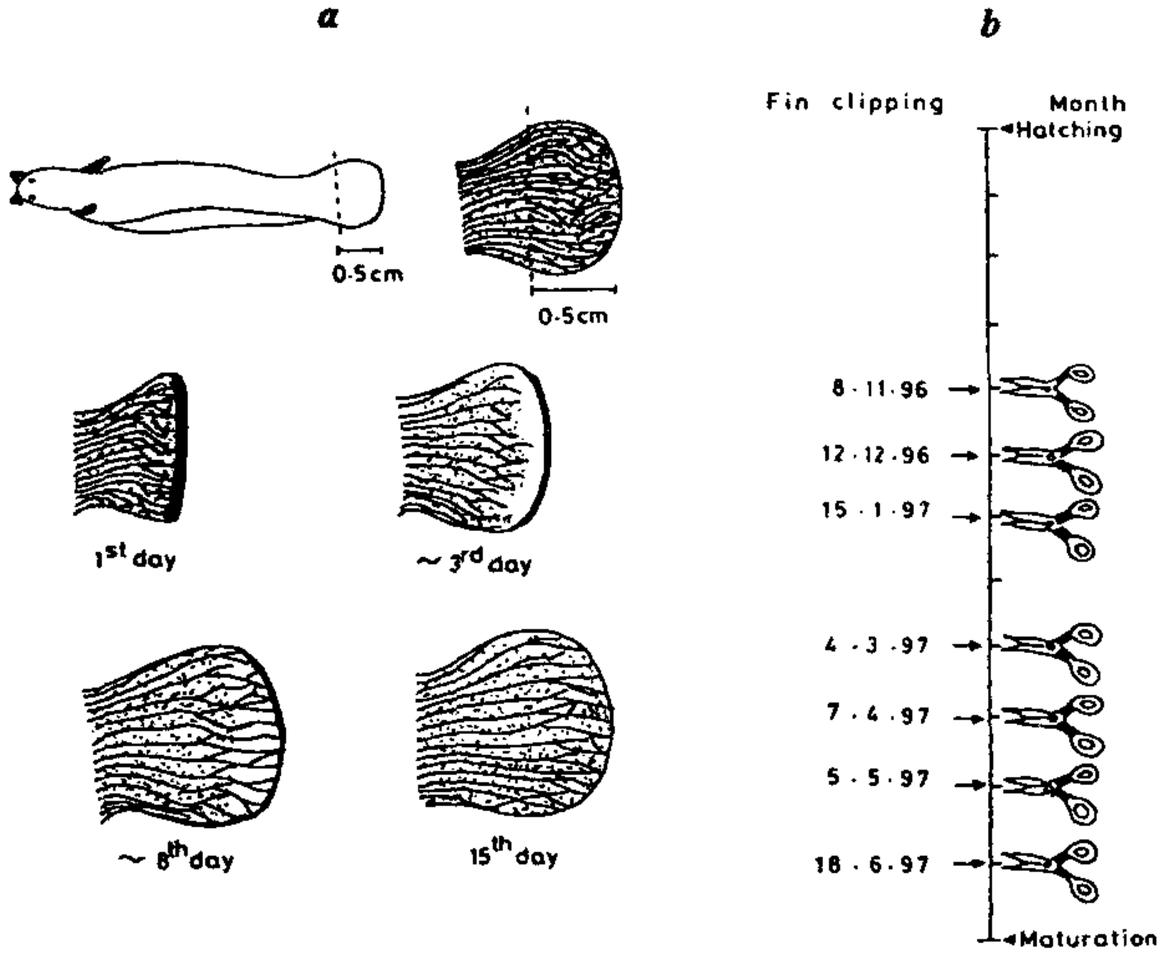


Figure 10. *a*, Schematic illustration of regeneration of tailfin in a representative individual of *Heteropneustes fossilis*; and *b*, date on which finclippings were obtained from the 10 experimental individuals during the successive seven clippings (from Sheela[99]).

transmission of GH gene into F_1 and in some cases F_2 progenies (e.g. ref. 104), clearly confirm the high frequency of mosaics among the founder transformants. Interestingly, the copy number of GH gene not only varied from tissue to tissue but also from individual to individual transformant catfish[88], zebrafish[96] and carp[100].

Consequent to this kind of mosaicism, transformants have also been shown to widely vary in size and in many cases suffer one or other deformity[99,105]. Deformities namely, presence of tumours, irregular fins, vertebral disorders[105], cranial deformity, opercular overgrowth[93], abnormal body shape, sharpened operculum, absence of body segment between anal and tail fins, degenerated fins, caudal and cervical ventral bent[99,105] are all different kinds of deformity suffered by transformants, whose zygotes were subjected to microinjection or electroporation. The reported increase in the copy number of the GH gene is likely to be the cause for destabilization of homeostasis during embryonic and post-embryonic development. Many authors have reported a progressive decrease in the frequency of putative transgenics from early embryonic stages to a year old adults[96]. Reports on the extrusion of transgene from cytoplasmic[106],

Table 9. Slot blot analysis of tissues drawn from the adult transformants[99]

		Tissue analysed									
Treatment	Transformants	Brain	Eyes	Skin	Heart	Muscle	Kidney	Gonads	Intestine	Gills	Liver
Electroporated with Zpβ*yp*GH	Y_1	+	+++	+	–	++	+++	–	++	++	++
	Y_2	–	+	++	+	++	+	++	++	–	++
	Y_3	+	+	+++	+	+++	+	–	+	+	+
	Y_4	–	–	–	–	–	+	–	+	–	–
Electroporated with Zpβ*n*GH	R_1	++	–	–	–	+	+++	+++	+++	++	+++
	R_2	++	–	++	+	–	–	+	–	+	–
	R_3	++	–	+	–	+	+	–	–	++	+
	R_4	+	–	+	+	–	–	–	–	+	–

Table 10. Transmission of growth hormone genes in transgenic fish

			Transmission	
Promoter	Coding sequence	Species	F_1	F_2
Mouse metallothionein	Human	Rainbow trout[125]	+	?
Mouse metallothionein	Rat	Rainbow trout[126]	+	?
Mouse metallothionein	Rat	Zebrafish[90]	+	+
Mouse metallothionein	Rainbow trout	Medaka[104]	+	+
RSV–LTR	Rainbow trout	Indian catfish[97]	+	?
RSV–LTR	Rainbow trout	Zebrafish[97]	–	?
RSV–LTR	Rainbow trout	Common carp[100]	+	?
Winter flounder *Zona pellucida*	Yellow porgy	Indian catfish[88]	+	?
Winter flounder *Zona pellucida*	Yellow porgy	Zebrafish[9]	+	?
Winter flounder *Zona pellucida*	Rainbow trout	Indian catfish[88]	+	?
Ocean pout	Rainbow trout	Zebrafish[9]	+	?
Sockeye salmon metallothionein B	Chinook salmon	Sockeye salmon[93]	+	?
Carp β-actin	Chinook salmon	Common carp[127]	+	?

Table 11. Comparative account on selected transgenic parameters in transformant catfish, whose eggs were electroporated with rtGHcDNA driven by different regulatory sequences[99]

Parameter	RSV-LTR-rtGHcDNA	ZpβrtGHcDNA	ZpβypGHcDNA
Regulatory sequence	Heterologous* (Viral promoter)	Homologous (Fish promoter)	Homologous (Structural gene changed)
Survival (hatching %)	15	57	55
Deformity (%)	> 30	27	29
G_0			
Integration (%)	15	100	40
Mosaicism	ND	++	++
Expression	+	+	+
F_1			
Integration (%)	16	100	50
Mosaicism	ND	+	+
Expression	ND	+	+

ND, Not determined.

nucleoplasmic[96] or chromosomal[108] sites are also known. Interestingly, not only is a single transgene extruded during successive cell cycle/subsequent generation[100], but also an entire set of chromosomes can be excluded in a triploid (e.g. carp[108]). It appears, therefore, that a species tends to maintain its integrity at the levels of gene, chromosome and ploidy by a mechanism, which is not clear.

In recent years, many efforts have been made to 'concentrate' the transgene by selectively breeding putative transgenic to achieve a stable germ-line transgenics. Here a major problem is the reduced fecundity and hatchability of such 'hybridized' eggs, which are the products of cross between putative transgenic and a non-transgenic[92].

Expression

With reference to expression, an aspect receiving attention during recent years is the overall role played by homologous or heterologous promoter of the transgene. Table 11 shows that both homologous and heterologous promoters ensured the expression in catfish, although the latter promoted a higher survival and integration frequency in both founder and F_1 progenies. Yet, the use of heterologus promoter is not desired for several reasons. A major reason is want of good expression (e.g. such transgenic rabbits, pigs and sheep failed to display the expected accelerated growth at an enhanced rate[109]).

Though its presence is detected, the expression of the transgene is wanting[79] or inadequate[110]. Relevant information available for fish and mammals suggests several reasons for inadequate or non-expression of the transgene. They may be summarized as follows: (1) Since the site of transgene integration is random, its expression may be strongly influenced by the site, at which it is integrated[111]. For instance, the integration at an undefined site may cause position effect, which may partly explain the lack of correlation observed between transgene copy number and level of expression[102]. As such a position effect can be alleviated by insulating the transgene (see ref. 111), an attempt to insulate fish GH may prove rewarding. (2) 'Gene silencing or inactivation' can be a second reason; for instance, integration of the transgene into its endogenous counterpart of the host genome by homologous recombination is known to inactivate the homologous transgene. This kind of 'co-suppression'[112] can be explained better by SSA recombination model[113], in which one allele is totally lost by recombination and the other is altered by injection. (3) The transgene may not join the host system due to its alienage for one or the other of the following, which may ultimately be responsible for the non-expression of the transgene: (i) nudity of the transgene, without the protein cover and compact packing, (ii) different methylation pattern of the transgene and (iii) difference in primary sequence[113]. (iv) Many cDNA constructs, which are known to express in *in vitro* cell culture system, do not express in a transgenic animal[114] and this kind of anomaly is implicated to genetic imprinting[115] which is not clear. Moreover, the transferred DNA upon integration into a mammalian genome is known to become inactivated by *de novo* methylation[114,115a]. Therefore, much remains to be done to ensure adequate expression of the introduced transgene in fishes.

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ACKNOWLEDGEMENTS. We acknowledge financial support of the Indian Council of Agricultural Research, University Grants Commission and Department of Biotechnology, New Delhi.