Towards an integration between chromosome set manipulation, intergeneric hybridization and gene transfer in marine fish culture

Colombo L., Bortolussi M., Argenton F., Dalla Valle L., Vianello S., Belvedere P., Barbaro A., Francescon A., Libertini A.

in

Bartley D.M. (ed.), Basurco B. (ed.).
Genetics and breeding of Mediterranean aquaculture species

Zaragoza : CIHEAM
Cahiers Options Méditerranéennes; n. 34

1998
pages 77-122

Towards an integration between chromosome set manipulation, intergeneric hybridization and gene transfer in marine fish culture

L. Colombo*, A. Barbaro, A. Francescon**, A. Libertini**, M. Bortolussi*, F. Argenton*, L. Dalla Valle*, S. Vianello* and P. Belvedere***
*Department of Biology, University of Padova
Via U. Bassi 58/B, 35131 Padova, Italy
**Institute of Marine Biology - CNR
Riva 7 Martiri, 1364, 30122 Venice, Italy

SUMMARY - In marine fish culture, production enhancement can be obtained not only through long-term projects of selective breeding to produce novel races and varieties according to the schemes of quantitative genetics, but also by exploiting the genetic gains arising from the application of innovative methods of biomanipulation at zygote, gamete, chromosome and gene levels, such as chromosome set manipulation, intergeneric and interspecific hybridization, sperm cryopreservation, nuclear transfer and transgenesis. The biotechnological approach is expected to provide additional benefits besides better performance in culture. First of all, since it requires the enforcement of measures ensuring an effective reproductive segregation of genetically modified fish from wild conspecifics, it may promote a policy to eliminate also the present genetic contamination of feral populations by domesticated escapees. Induction of triploidy and intergeneric hybridization may be used to generate gonadally sterile fish, which may grow faster than diploids after puberty for lack of the energy waste associated with gonadal maturation. Moreover, intergeneric hybrids may sometimes grow faster than the parental species, as in the case of certain sparid crossbreds. This fact seems to reflect a phenomenon of luxuriance, that is probably due to the attenuation of inhibitory regulations on growth whenever the underlying molecular mechanisms inherited from the parental species are poorly harmonizable within the hybrid. Another important advantage is the possibility of combining intergeneric hybridization, sperm cryopreservation and nuclear and gene transfer to diversify the appearance of marketed fish, while avoiding the costs and complications of setting up and selecting broodstocks of different species. In conclusion, aquatic biotechnology based on an integration of different techniques has the potential to transform radically the way the mariculture industry is presently organized, to favour new strategies for market control, and to foster a more strict co-operation between fish farms, research centres, control agencies and policy-making institutions in both developed and developing countries.

Key words: Polyploidy, gynogenesis, androgenesis, sex-control, hybridization, transgenesis.

RESUME - *"Vers une integration entre la manipulation chromosomique, l'hibridation intergénérique et le transfer de gènes en aquaculture marine". Dans le domaine de l'aquaculture marine l'amélioration de la production peut être poursuivie non seulement à travers des programmes à long terme de reproduction sélective, pour produire de nouvelles races et variétés selon les procédures de la génétique quantitative, mais aussi en exploitant les gains génétiques dérivant de l'application de méthodes innovatives de biomanipulation au niveau de zygote, gamète, chromosome et gène, telles que la manipulation chromosomique, l'hybridation interspécifique, la cryopréservation du sperme, le transfert nucléaire et la transgenèse. L'approche biotechnologique devrait fournir des bénéfices additionnels à une meilleure productivité. En premier lieu, étant donné qu'elle nécessite des mesures assurant une ségrégation reproductive effective des poissons modifiés génétiquement et de leurs congénères sauvages, elle peut promouvoir une politique visant à l'élimination de la contamination génétique des populations sauvages de la part d'individus domestiqués échappés. L'induction de la triploïdie et de l'hybridation intergénérique peuvent être utilisées pour produire des poissons stériles, qui peuvent présenter un taux de croissance plus élevé que celui des diploïdes*

étant donné le manque de perte d'énergie associée à la maturation des gonades. En outre, les hybrides intergénériques peuvent quelque fois ccroître plus rapidement que les espèces parentales, comme dans le cas de certains croisements entre sparidés. Ceci semble représenter un phénomène de luxuriance, qui est probablement dû à une atténuation des régulations inhibitrices sur la croissance chaque fois que les mécanismes moléculaires impliqués, hérités des espèces parentales, sont peu harmonisables chez l'hybride. Un autre avantage important est représenté par la possibilité d'associer l'hybridation intergénérique, la cryopréservation du sperme et le transfert nucléaire et des gènes pour diversifier l'aspect des poissons à commercialiser, tout en évitant les complications requises pour établir et sélectionner les stocks de reproducteurs des différentes espèces. En conclusion, la biotechnologie aquatique basée sur l'intégration de différentes techniques possède le potentiel de transformer radicalement l'organisation actuelle de l'industrie de la mariculture, pour promouvoir de nouvelles stratégies de contrôle du marché, et pour encourager une meilleure coopération entre élevages, centres de recherche, agences de contrôle et institutions politiques dans les pays développés ainsi que dans ceux en voie de développement.

Mots-clés : *Polyploïdie, gynogenèse, androgenèse, contrôle du sexe, hybridation, transgenèse.*

Fisheries, aquaculture and aquatic biotechnology

Fisheries

Since the late 1980's, capture fisheries landings have been levelling off at about 80-85 million metric tons (mmt) (FAO, 1994) and this productivity is presently regarded as near the upper limit. In the last decade, at least 20 major fisheries have collapsed around the world, including the North Atlantic cod fishery which was quite closely monitored and supposedly rationally managed (Schmidt, 1997). As of 1995, 6% of the target fish stocks were already depleted, 16% were overexploited and 44% fully to heavily exploited (Botsford *et al.*, 1997). In 1996, this state of affairs prompted the International Union for the Conservation of Nature (IUCN), in a controversial move, to add 118 marine fish, including overexploited food-fish species, such as Atlantic cod, haddock and bluefin tuna, to its Red List of threatened animals to give them greater visibility in future policy forums (Malakoff, 1997). This emerging awareness of the vulnerability of marine resources throws discredit on the complacent view that sea-fisheries are essentially inexhaustible because compensatory resilience of target fish stocks would be ensured by their great fecundity and vagility.

With a growing concern about ecosystemic impacts by fisheries (overfishing, destruction of benthic communities by dredging, trawling and other disruptive fishing practices, perturbation of food webs by the nonselective removal of target animals with consequent massive wastage of nonmarketable species, etc.) as well as about environmental impacts on fisheries (chemical pollution from coastal runoffs, eutrophication and algal blooms, oil spills, marine habitat fragmentation and degradation, introduction of foreign species, man-induced global changes on weather and ocean physics, etc.), there are discouraging perspectives about the capability of capture fisheries to satisfy the anticipated increase in the demand for seafood by the expanding human population. Actually, improvements in fishery management are expected to rely on more precautionary approaches to fishing effort and harvesting pressure, implementation of marine reserves and temporary "no-take" zones, enforcement of moratoria, adoption of more selective fishing gears and other remedial measures that are likely to sacrifice short-term economic gains in favour of more sustainable, though diminished catches (Botsford *et al.*, 1997).

In the Mediterranean Sea, the situation is particularly critical owing to its relatively small extension, moderate overall bioproductivity and great number of countries with gravitating fisheries. A regulation from the European Community (27-06-1994, n. 1626/94) enforces technical measures to preserve the Mediterranean fishing resources. These measures that establish the minimal net meshes and fish sizes for fishing activity should be complemented in the future by co-operative agreements among all interested nations about more effective approaches for fish stock assessment and conservation.

Aquaculture

While many capture fisheries are facing declining or stagnant harvests, there is a widespread confidence that a growing portion of the necessary production to secure future foodfish availability will eventually be supplied by aquaculture. This expectation is based on the impressive figures of total world aquaculture growth in the past decade, with a doubling of landings from 10.4 mmt in 1984 to 22.6 mmt in 1993 (FAO, 1995). In this scenario, the production figures of marine fish culture in the Mediterranean Basin are comparatively small, but their increment is nonetheless striking as seabass/seabream farming has increased six-fold in the period 1990-1995 reaching about 42,000 tons produced by almost 500 intensive enterprises. The share of EU countries has been steady around 80% throughout this period, supported by a strong incentive policy from the European Commission (Harache and Paquotte, 1996).

On a global scale, aquaculture is currently one of the fastest growing food production systems, increasing at an annual rate of 9% compared to 2.8% for total livestock meat production (Tacon, 1996). It is estimated that in order to maintain current levels of foodfish consumption, global aquaculture production should be doubled by the year 2020 and trebled by 2040 (New, 1997a). Whether this anticipation is an achievable goal, however, is still an open question.

Aquaculture is an extremely heterogeneous industry devoted to the farming of a multitude of animal and plant species and characterized by a marked technological polarization. In developing countries (which account for 85% and 86.7% of total and finfish global aquaculture production, respectively) (Tacon, 1995), traditional low-cost, low-stocking density, subsistence-oriented polyculture technologies are mainly employed, whereas in developed countries high-cost, high-stocking density, profit-oriented monoculture technologies prevail.

Although both farming systems are at present economically viable in their respective areas, increasing competition for land and water as well as broadening of international foodfish trading are promoting a trend towards greater intensification of farming systems in developing countries. On the other hand, commercial competition for feed resources, especially fish meal and fish oil, and rising protest against environmental impacts by aquafeed usage and farm effluents are demanding less negative energy and nitrogen balances in nutrient conversion by cultured species and better environmental compatibility of aquafarms in developed countries. These tendencies are likely to reduce some of the differences in technological approaches and management strategies between developing and developed countries.

A greater technological homogeneity in the aquaculture industry is likely to facilitate technology transfers, farmer training and convergence of action towards the main obstacles that still lie along the path of an announced prodigious growth. The most crucial ones are the incidence of disease outbreaks, the effects of market price fluctuations or decline on enterprise operativeness and opposition by opinion groups and lobbies that are rightly concerned about environmental protection, conservation of biodiversity and animal welfare.

It is generally recognized that research intensification and technological development can appropriately address these problems. Less consensus, however, exists about the need to integrate aquatic biotechnology more deeply in the context of aquaculture than it is currently proposed.

Aquatic biotechnology

The realization of the role of aquatic biotechnology means that aquaculture in the coming decades should undergo a radical transformation driven by both engineering innovations (e.g., offshore submersible cages for mariculture monitored by remotely-operated, camera-equipped underwater vehicles; inland water-recirculating systems for fish fattening coupled to energetically complementary cultures, such as hydroponics, macroalgae or sponge cultivations; on-line process automation etc.) and bioengineering of aquatic organisms. In this scenario, the aquacultural products of the future will be raised with innovative methods and will appear somehow different from the products offered today to the consumers.

Probably this vision may appear controversial, if not hazardous, not only to those that feel oppressed by technological progress and viscerally oppose biotechnology, or simply live by the NIMBY (not in my backyard!) creed, as acutely pointed out by New (1997b), but also to those that, with different roles and positions, wish to convey a more reassuring image of aquaculture to the general public. Mass media often depict biotechnology with a scaring profile or attributes unrealistic powers to it, so that it is mostly perceived as an unnecessarily manipulative and intrusive option.

Actually, the development of biotechnological aquaculture is not a postponable option but rather an indispensable course to be taken if the projected feats of aquaculture must be something more than wishful thinking. The objection that the incorporation of biotechnology into aquaculture is likely to exacerbate opposition by the anti-aquaculture lobby and further confuse the layman's opinion can indeed be reversed, if one considers that biotechnology preliminarily requires a stringent validation of its technical approaches in terms of environmental safety and biological harmlessness. Thus, it may actually provide solutions to still open problems about ecosystemic impacts by aquaculture.

For instance, the enforcement of gonadal sterility in genetically manipulated fish to segregate them reproductively from wild conspecifics could be adopted as a general measure to avoid any genetic meddling between farmed escapees and wild fish. The search for viable alternatives to offset market saturation other than the recourse to new species for cultivation may also be pursued through hybridization, sperm cryopreservation and chromosome set manipulation. Molecular biology techniques

provide faster and cheaper diagnostic assays for aquatic pathogens (Argenton *et al.*, 1996b) and recombinant DNA technology may be adopted to confer immunocompetence and disease resistance (recombinant vaccines, antisense transgenes, DNA immunization, etc.).

Inclusion of ethical issues into the guidelines for sustainable aquaculture, as suggested by New (1997b), is a sensible step to defuse the mounting impatience against the possible adverse consequences of aquaculture expansion. As to the technological upgrading to reduce sensitive interfaces and increase efficiency, the best strategy is to accelerate progress along multiple lines. These should include better farm design and management with appropriate measures for disease prevention and containment, the genetic improvement of broodstock according to the principles of quantitative genetics (selective breeding and crossbreeding of inbred lines), as well as productive improvements by chromosome and genetic engineering. There is only a short lapse of time ahead of us to solve the present emergencies and endow the aquaculture sector with the means and technologies to attain its goals. All opportunities must be explored and compared without preconceptual exclusions.

In this paper, an effort is made to outline the main advantages and problems associated with the application of the techniques of chromosome set manipulation, sex control, intergeneric hybridization and gene transfer in fish culture, with particular reference to cultured Mediterranean marine teleost fish. The complementarity and integrability of these techniques will be also discussed and new data from our laboratory briefly presented.

Chromosome set manipulation

In fish culture, the techniques of chromosome set manipulation can be used for two different purposes: either to induce polyploidy, essentially to obtain triploid and tetraploid fish, or to reproduce fish by uniparental chromosome inheritance to obtain gynogenetic and androgenetic fish. The main aspects of this "chromosome engineering" have been reviewed by several authors (Thorgaard, 1983; Chevassus, 1987; Chourrout, 1987; Thorgaard and Allen, 1987; von Lukowicz *et al.*, 1990; Thorgaard *et al.*, 1992; Lu *et al.*, 1993; Purdom, 1993b; Horvath and Orban, 1995; Colombo *et al.*, 1996).

Two points are worth considering: firstly, changes from diploidy to polyploidy and uniparental reproduction may occur naturally in teleosts. For instance, salmonids have apparently undergone a tetraploidization event in their ancestry (Johnson *et al.*, 1987) and spontaneous triploids and tetraploids are sometimes found within diploid fish populations (Schultz, 1980; Arai *et al.*, 1995). A round or two of tetraploid evolution is surmised even in the piscine ancestors of mammals (Ohno, 1993). Gynogenesis is the sole mode of reproduction of some populations of the crucian carp (*Carassius auratus gibelio*) and several species of the family Poecilidae, while spontaneous gynogenesis or androgenesis is sometimes observed in crosses of different fish species (cf., Thorgaard, 1983). Secondly, the techniques of chromosome set manipulation can be usefully applied to complement other techniques, such as interspecific hybridization, artificial speciation, genetic engineering, sex control, population control, selective breeding, pure line or clone crossbreeding and sperm cryopreservation.

Induction of polyploidy

The main procedures for the induction of polyploidy in fish consist in either the blockage of the resumption of the second meiotic division in the fertilized oocyte with consequent retention of the second polar body (meiotic block), or the suppression of the mitotic division at the first embryonic cleavage with consequent fusion of the two blastomere genomes (mitotic block). Since at syngamy, during normal fertilization, a diploid secondary oocyte is fused with a haploid spermatozoon, the retention of the haploid chromosome set of the second polar body induced by the meiotic block will result, at karyogamy, in a triploid chromosome complement. On the other hand, the doubling of the diploid chromosome set of the zygote caused by the mitotic block will result in a tetraploid status.

Several methods can be utilized to induce meiotic or mitotic blocks, such as a thermal shock (heat shock at a sub-lethal temperature or cold shock at 0-4°C), a hydrostatic pressure shock, electrofusion (electric field-induced cell fusion) or exposure to chemical fusogens (e.g., nitrous oxide, cytochalasin B, polyethylene glycol). All these treatments must be applied just before the presumptive time of meiotic resumption or mitotic division. Thermal and pressure shocks are usually preferred to the other methods since they are potentially less traumatic or toxic (Palti *et al.*, 1997). Ploidy levels can be assessed by a variety of techniques, including measurement of erythrocyte nuclear or cellular volumes, nucleolar counting, chromosome counting, and DNA content determination by flow cytometry (cf., Chourrout, 1987; Purdom, 1993b; Zhang and Arai, 1996).

Triploidy

Interest in triploid fish arises from the fact that they are mostly sterile owing to either a lack of gonadal development beyond a rudimentary differentiation stage with consequent gametogenetic failure (gonadal sterility), or lack of gamete fertility due to meiotic abnormalities caused by the odd number of chromosome sets (gametic sterility) (Yamazaki, 1983). The severity of gonadal development inhibition varies with species and sex (Bramick *et al.*, 1995). Generally, triploidy inhibits more ovarian than testicular development, because it does not interfere with the multiple waves of spermatogonial mitotic divisions. Moreover, secondary sexual characters and sexual behaviour are not always suppressed in males (Thorgaard, 1983).

There are a number of possible benefits associated with gonadal sterility, such as:

(i) In relation to growth and survival: (a) faster growth rate of triploids after the normal time of puberty of diploids, as the former do not shunt energy from somatic growth into gonadal maturation (Bramick *et al.*, 1995); (b) increased longevity and growth to trophy size in released fish that normally experience high mortality at the time of spawning, like many salmonids; (c) lack of the decrement in the bactericidal activity of serum observed in diploid fish during gonadal maturation and spawning (Yamamoto and Iida, 1995); and (d) intensification of flesh colour in salmonids fed canthaxanthin at the end of the fattening stage, when this step is performed during the normal breeding season (Choubert and Blanc, 1985).

(ii) In relation to population control: (a) population control in case of prolific species

which tend to overpopulate confined water bodies; and (b) population control in case of introduced allochthonous species.

(iii) In relation to hybridization: (a) enhanced survival of certain interspecific and intergeneric hybrids, whenever genome incompatibility is mitigated by the double maternal gene dose (Scheerer and Thorgaard, 1987); (b) prevention of introgressive hybridization, when a restocked variety or species can interbreed with a threatened sympatric variety or species; (c) avoidance of backcrossing between partially fertile interspecific hybrids and their parental species; and (d) reproductive segregation of fertile interspecific hybrids.

(iv) In relation to transgenesis: reproductive segregation of genetically engineered fish from wild conspecifics.

The main drawbacks in the exploitation of induced-triploidy are: (i) difficulty in obtaining 100% triploid fish by meiotic block due to rejection of the sperm genome or failure of second polar body retention (Thorgaard *et al.*, 1995); (ii) lower prepubertal growth rate of triploids in some species, especially when triploids are cultured communally with diploids (Carter *et al.*, 1994; Tave, 1993); and (iii) lower respiratory capacity of triploids due to a less favourable surface/volume ratio in red blood cells (Aliah *et al.*, 1991) and, at least in rainbow trout, *Oncorhynchus mykiss*, a lower blood haemoglobin concentration leading to macrocytic anaemia (Yamamoto and Iida, 1994) and dimished survival and growth at chronic high temperatures (Ojolick *et al.*, 1995).

Tetraploidy

Tetraploidy has been less intensively investigated than triploidy and its potential advantages are still to be fully explored. Newly arisen tetraploids are often less viable than diploids and triploids (Goudie *et al.*, 1995) and fertility may be reduced in autotetraploids because of unbalanced disjunction of chromosomes after meiotic multivalent pairing.

Theoretically, induced tetraploid fish may be exploited:

(i) In relation to genetics: to produce superheterozygous fish with up to four different alleles per locus and increased enzyme multiplicity.

(ii) In relation to two-step chromosome set manipulation: (a) to generate autotriploid progenies with a lower level of homozygosity when tetraploid fish are crossed with isospecific diploids; and (b) to obtain androgenotes by fertilizing genetically inactivated eggs with diploid sperm from autotetraploid males (Thorgaard *et al.*, 1990).

(iii) In relation to hybridization: (a) to create new tetraploid hybrid species by polyploidization of interspecific hybrids into fertile allotetraploids (artificial speciation); (b) to produce sterile allotriploids when an autotetraploid is crossed with a diploid mate of a different, genetically compatible species, or alternatively when an allotetraploid is crossed with a diploid mate of one of its original parental species; (c) to obtain sterile triple-hybrid allotriploids by crossing an allotetraploid with a diploid mate of a species different from the parental species of the allotetraploid.

Although polyploidization is recognized as a basic mechanism for fast speciation in nature and a dynamic force in evolution (Soltis and Soltis, 1995), it should be noted that bringing into existence a new hybrid species by induced allotetraploidy entails the risk of ecosystemic impacts on wild fish populations and other aquatic organisms, especially when the hybrids possess great invasiveness and good fertility. Regulations about their containment must, therefore, be assimilated to those for either allochthonous species (as synthetic species are foreign to the local fauna) or genetically modified organisms (as two phylogenetically distinct sets of genes are recombined into a novel perpetuating germline).

Uniparental chromosome inheritance

Fertilization using either eggs or sperm whose genetic material has been inactivated by radiation gives rise to haploid gynogenetic and androgenetic fish, respectively. Ionizing radiation (γ-rays and X-rays from ^{60}cobalt and radium sources; dose range: 30-150 krad) shatters DNA, whereas short-wave ultraviolet light (UVc; wavelength: about 254 nm; dose range: 3-40 kerg mm^{-2}) destroys DNA by thymine dimerization. Though less penetrating, UVc light is preferred to ionizing radiation because: (i) it does not require hazardous machinery unavailable at fish farms; (ii) it does not leave supernumerary chromosomal fragments, residual from the irradiated gamete, inside the zygote nucleus, where they can be transcriptionally active and replicated during embryogenesis; and (iii) it results in better morphology of surviving haploid embryos or larvae (Chourrout, 1986, 1987). Ionization radiation is, however, needed to treat opaque yolky oocytes or large volumes of undiluted milt.

In teleosts, haploidy is usually incompatible with survival beyond embryonic or early larval stages, though a low viability with heavy abnormalities has been observed in haploid tilapia (Varadaraj, 1993). Diploidization of the maternal chromosome complement in gynogenotes can be carried out by either meiotic block (*meiogynogenesis*) or mitotic block (*mitogynogenesis*), using the same techniques adopted for polyploidization (Palti *et al.*, 1997). Androgenotes, instead, can be diploidized exclusively by mitotic block.

To confirm uniparental chromosome inheritance, special control measures can be taken in the selection of the parent whose gametes are genetically inactivated. This may be characterized by a visible dominant trait which is absent in the other mate and shows up in biparental progeny. Alternatively, it may belong to a different species whose hybrids are phenotypically divergent or unviable in the diploid and triploid conditions (Chourrout, 1987).

Meiogynogenesis

Meiogynogenotes are in general only partially homozygous, their heterozygosity level depending upon the frequency of gene recombinations caused by odd numbers of crossovers between homologous chromatids. Despite their lack of complete homozygosity, meiogynogenetic fish can be utilized for a variety of purposes, such as:

(i) In relation to triploidy induction: to define the best conditions for triploidy induction as indicated by the best survival of gynogenetic progeny after meiotic block.

(ii) In relation to sex control: (a) to explore the genetic sex determination mechanism and the influence of environmental factors, if any, on the final sexual phenotype (Mullerbelecke and Horstgenschwark, 1995); (b) to produce directly all-female progenies in species with genetically determined sex, female homogamety and better performance in culture of females than males (Palti and Thorgaard, 1997); (c) to overcome the difficulty in the discrimination of pseudomales (genetic females inverted into phenotypic males) from genetic males, whenever meiogynogenotes are all-females, thus yielding, after sex reversal, all pseudomales; the latter can be crossed to normal females to generate all-female progenies (monosexualization of fish stocks) (Thorgaard, 1983; Pandian and Varadaj, 1990).

(iii) In relation to genetics: (a) to discharge part of the genetic load of harmful recessive alleles from a founder broodstock gene pool, because offspring homozygous for such alleles would be phenodeviant and defective and, thus, discarded; (b) to facilitate mapping of genes relatively to their centromeres, especially in species with many chromosomes, because the closer a locus to its centromere, the lower the frequency of heterozygotes in the meiogynogenetic progeny (Thorgaard *et al.*, 1983); and (c) to estimate the degree of conservation of gene arrangements and differences in gene recombination rates among related species (Thorgaard, 1983).

Mitogynogenesis

Mitogynogenotes are completely homozygous, since their genome derives from a mitotically duplicated haploid chromosome set. Their viability, sex and fertility have been explored in experimental fish models, such as the zebrafish, *Danio rerio* (Streisinger *et al.*, 1981) and the medaka, *Oryzias latipes* (Naruse *et al.*, 1985), as well as in rainbow trout (Quillet, 1994). Their potential use in fish culture has been examined in the common carp, *Cyprinus carpio* (Komen *et al.*, 1991), flounder, *Paralychthys olivaceous* (Hara *et al.*, 1993), ayu, *Plecoglossus altivelis* (Taniguchi *et al.*, 1996), gilthead seabream, *Sparus aurata*, and European seabass (named seabass below), *Dicentrarchus labrax* (Colombo *et al.*, 1996).

Several possible applications of mitogynogenesis can be envisaged, such as:

(i) in relation to genetics: (a) to free completely a broodstock gene pool from its load of deleterious recessive alleles, because all of them will be manifested in the offspring and, thus, culled; (b) to assess phenotypic variation due to environmental factors in the estimate of genetic heritability (h^2) for quantitative traits, as clonal fish have no genetic variation (del Valle *et al.*, 1994; Tsujimura and Taniguchi, 1996); and (c) to study the influence of homozygosity on developmental stability, as measured by fluctuating bilateral asymmetry for meristic traits (fin rays, gillrakers on the first branchial arches, mandibular pores etc.) (Leary *et al.*, 1985; Taniguchi *et al.*, 1990).

(ii) In relation to cloning: (a) to produce fish clones whenever F_1 mitogynogenotes,

which are homozygous but not mutually isogenic due to maternal allele recombinations by meiotic crossovers, are reproduced gynogenetically yielding, at F_2, an isogenic homozygous offspring (autozygous clone) from each mitogynogenetic clonal progenitor (Young *et al.*, 1996); (b) crossing of normal and sex-inverted fish from two different clones to obtain, at F_3, heterotic interclonal hybrids (allozygous clone) with enhanced performance in culture (Young *et al.*, 1995); (c) crossing of several clones to form a multiclonal broodstock, which can be subjected to intense selective pressure without any risk of inbreeding depression.

(iii) In relation to histocompatibility and disease resistance: (a) to analyse the histocompatibility gene system in fish by determining the fate of skin allografts exchanged among different clones (Komen *et al.*, 1990); and (b) to discriminate innate disease resistance by challenging a set of clones with different pathogens (Wiegertjes *et al.*, 1996); certain clones of common carp exhibit a genetically predetermined low antibody response and high susceptibility to infection (Wiegertjes *et al.*, 1995).

(iv) In relation to transgenesis: to achieve homozygosity of transgenes in the mitogynogenetic progeny of clonal, or even non-clonal, transgenic fish with an initial hemizygous or mosaic transgene distribution in order to set up stabilized transgenic fish lines with 100% transmission of the transgenic trait.

It should be note that, in the course of time, isogenicity may not be maintained in autozygous clones in the presence of an intense spontaneous mutation rate. In zebrafish, an isogenic clone became polyallelic at a malate dehydrogenase locus after 10 bisexual generations (Buth *et al.*, 1995). So, recloning may be necessary to ensure genetic uniformity that may be checked by DNA fingerprinting (Young *et al.*, 1996).

Fragment gynogenesis

Fragment gynogenesis, consisting in the inclusion of paternal chromosome fragments, inherited from γ-ray-treated sperm, in the gynogenome, has been attempted in rainbow trout (Disney *et al.*, 1987, 1988). This approach would be equivalent to a chromosome-mediated gene transfer and may be exploited: (a) to map genes on chromosomes, especially in case of hybrid gynogenotes; (b) to aid in the identification of major genes for important traits, like sex determination; and (c) to create auto- or allotransgenic fish for multiple genes donated by iso- or allospecific males, respectively, whenever centromere-bearing fragments are diploidized by mitotic block and perpetuated in the descendants.

Androgenesis

Similarly to mitogynogenesis, diploid androgenesis, which involves the genetic inactivation of the egg by irradiation with doubling of the fertilizing sperm chromosome set, is also a fast method for producing fish clones in only two generations. This is much faster than the production of pure lines by multiple sib-mating. Egg irradiation, however, may damage not only nuclear DNA but also mitochondrial DNA and ooplasmic RNAs which are essential for embryonic development. Low survival of androgenotes has been reported in various species

(Scheerer *et al.*, 1991; Bongers *et al.*, 1995), with some improvement when diploid sperm from tetraploid males was used for androgenesis (Thorgaard *et al.*, 1990). Moreover, low fertility was observed in both androgenetic males and females of rainbow trout (Bongers *et al.*, 1995) and viability of YY individuals must be established in each species with XY sexual chromosomes.

Despite these drawbacks, androgenesis has two important applications:

(i) In relation to sex control: (a) to produce directly all-male progenies in species with genetically determined sex, male homogamety, and better performance in culture of males than females (Myers *et al.*, 1995); analogously to meiogynogenotes, these androgenotes may then be sex-inverted into pseudofemales to produce all-male stocks when crossed with normal males (Pandian and Sheela, 1995); and (b) to obtain YY males in case of male heterogamety (XY) that may be utilized to generate an all-male progeny when crossed with normal females (XX) (Mair *et al.*, 1995).

(ii) in relation to sperm cryopreservation: to ensure recovery of fish clones or natural ecotypical strains from their cryopreserved sperm stored in gene banks for perpetuation or conservation genetics programs (Scheerer *et al.*, 1991; McAndrew *et al.*, 1993).

Furthermore, androgenesis may provide a tool for artificial speciation in several still theoretical ways, such as: (a) fertilization of genetically inactivated eggs with sperm from partially fertile diploid interspecific hybrids and subsequent diploidization by mitotic block; any viable fish could then be used to found new diploid hybrid species, each receiving variable genomic contributions from the original parental species; and (b) fertilization of genetically inactivated eggs with sperm from another species and subsequent diploidization to give rise to nuclear-cytoplasmic hybrids. Containment of these fish should be equivalent to that of allotetraploids outlined above.

Chromosome set manipulation in marine fish culture

Chromosome set manipulation has been investigated and applied to increase productivity mainly in salmonids, cyprinids, cichlids and ictalurids (von Lukowicz *et al.*, 1990; Thorgaard *et al.*, 1992; Purdom, 1993b; Volckaert *et al.*, 1994a; Bongers *et al.*, 1995; Bramick *et al.*, 1995; Horvath and Orban, 1995; Myers *et al.*, 1995). Research on farmed marine fish species, mostly belonging to the families Pleuronectidae (Hoornbeek and Burke, 1981; Purdom, 1993b), Moronidae and Sparidae, has generally begun more recently. The first preliminary report on ploidy manipulation and sex control in seabass by Carrillo and coworkers appeared in 1993. Chromosome set manipulation in gilthead seabream was reported for the first time by Francescon *et al.* in 1994. Further progress in both species has been recently reviewed by Colombo *et al.* (1996).

Meiotic and mitotic blocks have been carried out with good yields (mostly >75% and up to 100%) in both seabass and seabream using cold shocking (0-2°C for 13-20 min at 3-5 min post-fertilization, PF) and pressure shocking (58.8-88.2 MPa for 3-

7 min at 40-90 min PF), respectively. Meiotic block was used to produce triploids and meiogynogenotes, while mitotic block gave rise to tetraploids and mitogynogenotes (Colombo *et al.*, 1995, 1996; Barbaro *et al.*, 1996). As shown in Fig. 1, the ploidy level of haploid, normal diploid, triploid and tetraploid seabream was evidenced by their respective karyotypes and was correlated with the highest number of nucleoli per nucleus in embryonic or larval cells. Analogously, Fig. 2 shows the karyograms of similarly manipulated seabass.

The efficacy of cold shocking in the mass production of triploid seabass was also confirmed by Gorshkova *et al.* (1995), Curatolo *et al.* (1996), Felip *et al.* (1997) and Barbaro *et al.* (1998). On the other hand, heat shocking was found to be poorly effective (25% yield) at 29°C for 25 min at 15 min PF (Zanuy *et al.*, 1994) but very effective (83% yield) at 32°C for 10 min at 13 min PF, although 5-9% tetraploids were also concomitantly produced (Curatolo *et al.*, 1996). Low percentages of triploidy in seabass were observed with pressure shock (58.8 MPa for 2-3 min at 5 min PF) (Zanuy *et al.*, 1994) and cytochalasin B treatment (0.5-1 mg/l at 5 min PF) (Curatolo *et al.*, 1996). Among other species of the family Moronidae, triploidy was induced in striped bass, *Morone saxatilis* (Hallerman, 1994), and hybrid meiogynogenotes were obtained from eggs of sunshine bass (female *Morone chrysops* x male *M. saxatilis*) fertilized with inactivated sperm from white perch (*Morone americana*) (Leclerc *et al.*, 1996).

In *S. aurata*, 100% triploidy was reportedly induced by heat shocking (34°C for 10 min at fertilization) (Garrido-Ramos *et al.*, 1996). Heat shocking at a rather high temperature (36.5-37.3°C) was also successfully applied to gilthead seabream eggs fertilized with normal and UV-inactivated sperm of Japanese red seabream (named red seabream below), *Pagrus major*, giving rise to meiogynogenetic and triploid hybrid offspring (Gorshkova *et al.*, 1995). Retention of the second polar body was accomplished in red seabream obtaining triploids by cold shocking (Sugama *et al.*, 1992) and meiogynogenotes by both cold and heat shockings (Sugama *et al.*, 1990a,b).

Androgenesis has not been attempted so far in *D. labrax* and *S. aurata* despite the fact that the transparency of ovulated eggs in these species may allow their genetic inactivation by exposure to UV light instead of ionizing radiation.

To provide clues for future research, the following points and new results are worth considering:

(i) Thermal shocks are ineffective in blocking the first mitotic division in both seabass and seabream (Barbaro *et al.*, 1996), as also found in red seabream (Sugama *et al.*, 1990a,b).

(ii) Tetraploids and mitogynogenotes of seabass and seabream show lower survival at hatching (Colombo *et al.*, 1996) and during subsequent development as compared to triploids and meiogynogenotes. In particular, when a lot of seabass, subjected to mitotic block and containing 56% tetraploids and the rest diploids at hatching, was raised for ten months, it was found that tetraploids were reduced to 33% after one month and were no longer present at eight months, leaving only surviving diploids (Barbaro *et al.*, 1998). Therefore, purely tetraploid seabass should

be grown in order to establish whether they are outcompeted by diploids or merely short-lived.

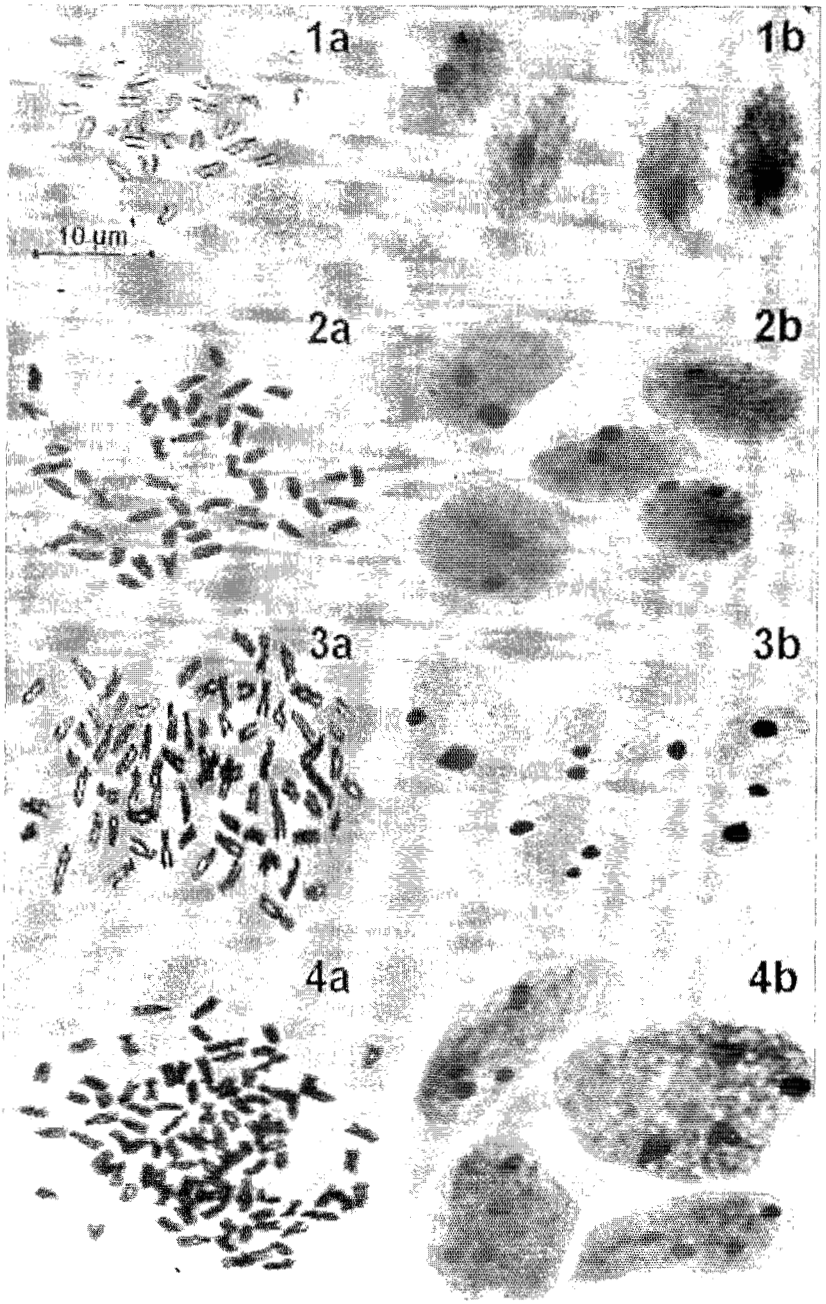

Fig. 1. Metaphase chromosome plates (1a-4a) and nuclei of embryonic or larval cells with nucleoli evidenced by silver nitrate staining (1b-4b) obtained from gilthead seabream (*Sparus aurata*) haploid (1a,b), normal diploid (2a,b), triploid (3a,b) and tetraploid (4a,b) individuals.

(iii) Triploid seabass appear to be sterile. In triploid females, the weight of the ovary at 2 years of age was only 14% of that of control diploids. The ovigerous lamellae contained mainly oogonial nests with only few scattered primary

previtellogenic oocytes, indicating that initiation of meiosis was impaired. In triploid males, the weight of the testis was significantly lower than that of diploids at 3 but not at 2 years of age. In controls, the testicular lobules were full with cysts at all stages of spermatogenesis, while in triploids germ cells were represented only by scattered spermatogonia and more abundant primary spermatocytes (Colombo *et al.*, 1996; Barbaro *et al.*, 1998). Thus, triploidy in seabass hinders the onset of meiosis but not gonial proliferation in both sexes, as reported also in red seabream (Sugama *et al.*, 1992).

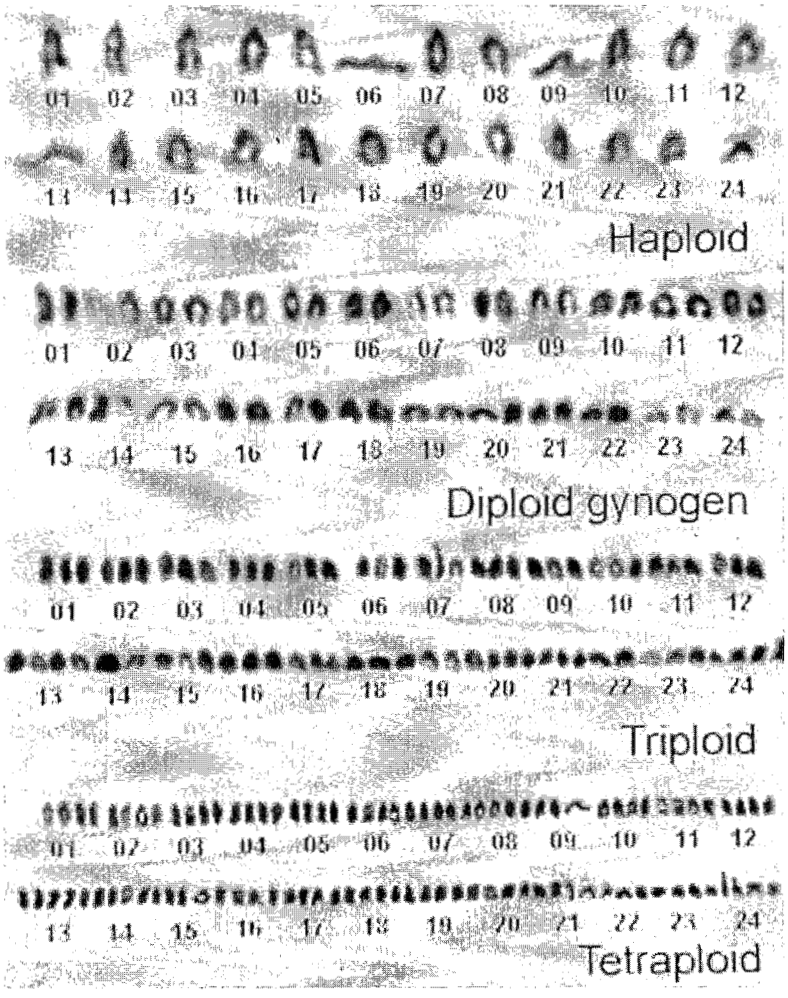

Fig. 2. Karyograms derived from metaphase chromosome plates obtained from European seabass (*Dicentrarchus labrax*) haploid, meiogynogenetic diploid, triploid and tetraploid individuals.

(iv) Throughout the prepubertal period, the growth rate of triploid seabass remained significantly lower than that of diploids in two distinct trials. In the first one, the mean body weight at 20 months was 73% of that of normal diploids (115 *vs.* 158 g, n = 110; $P<0.05$); in the second one, at 31 months, it was 66% of that of meiogynogenetic diploids (241 *vs.* 365 g, n = 72 and 36, respectively; $P<0.05$) (Barbaro *et al.*, 1998). At 3 yr of age, however, the divergence between triploids and

diploids became no longer significantly different (1129 *vs.* 1241 g) (Colombo *et al.*, 1996). This suggests that, since sexual maturity is dependent upon size (Roblin and Bruslé, 1983) and is usually attained at the end of the 2nd year in males and of the 3rd year in females, the culture of triploid seabass would be convenient just to produce fish of big market size or suitable for filleting. In red sea bream, no difference in growth rate was observed between diploid and triploid full-sibs (Sugama *et al.*, 1992).

(v) During ongrowing for 16 months, the mean body weight of meiogynogenetic seabass was comparable to that of normal diploids but was significantly lower at 20 months of age (126 vs. 158 g, n = 25 and 110, respectively; $P<0.05$), though still greater than that of triploids (see above) (Barbaro *et al.*, 1998). Actually, the difference merely in the last weight values may be fortuitous and cannot be regarded as a conclusive proof of a lower performance by meiogynogenotes. In red seabream, no significant difference was observed in growth rate between meyogynogenetic and normal diploids up to 6 months of age (Sugama *et al.*, 1990b).

(vi) The possibility of spontaneous diploidization in a small percentage of untreated gynogenotes by retention of the second polar body has been verified in both seabass and seabream (Colombo *et al.*, 1996). A similar phenomenon has been described also in the common carp (Cherfas *et al.*, 1995).

(vii) Despite the fact that seabass meiogynogenotes bear only maternal chromosomes, they can spontaneously differentiate into males. This observation, coupled to the fact that cultured diploid seabass often display sex ratios strongly skewed in favour of males (3:1) (Carrillo *et al.*, 1995), but sometimes also in favour of females, hint at a more complicated mechanism of sex determination in this species than earlier suspected. As summarized in Table 1, in four different experiments at three locations, the sex ratio of the control diploids ranged from 4:1 in favour of females at Pellestrina (Barbaro *et al.*, 1998) to 3.5:1 in favour of males at Eilat (Gorshkova *et al.*, 1996), but included also a sex ratio of 1:1 at Monfalcone (Colombo *et al.*, 1996). These data seem to point at a thermal influence on sex determination because fingerlings at the hatchery of Pellestrina are raised at a lower water temperature than that at Eilat, while Monfalcone stays in between as it utilizes the effluents of a power station. Significantly, in the Nile tilapia, *Orechromis niloticus,* exposure of fry to high temperature was found to increase the proportion of males (Baroiller *et al.*, 1996). However, the observed sex ratios of the meiogynogenotes do not conform to the assumption that males prevail at higher water temperatures. As a matter of fact, while meiogynogenotes differentiated more into females, the relative abundance of males was greater at Pellestrina than Eilat, and thus it was inversely related to the water temperature. It is really surprising that, at Pellestrina, there were less females among the meiogynogenotes than in control diploids. Recently, in a lot of seabass meiogynogenotes raised at a fish farm in Sicily, 58% were found to be males (P. Benedetti, personal communication).

A differentiation of gynogenotes into males has been reported also in the Nile tilapia (Mullerbelecke and Horstgenschwark, 1995). In this species, a dual system of sex chromosomes with homogametic and heterogametic individuals of the same gender has been proposed (Trombka and Avtalion, 1993). In another teleost, *Leporinus elongatus,* the sexual heterochromosomes may recombine yielding

atypical sex karyotypes and unbalanced sex ratios (Baroiller *et al.*, 1996). Clearly, more data are necessary to disentangle genetic from environmental effects and, at present, the mechanism of sex determination of seabass remains obscure, as discussed by Colombo *et al.* (1996). An interesting experiment would be to establish the sex ratio in F_2 meiogynogenotes derived from female meiogynogenotes (or even better mitogynogenotes) and raised under different environmental conditions.

Table1. Percentages of females and males in stocks of meiogynogenetic European seabass, *Dicentrarchus labrax*, produced at different sites in Italy and Israel as compared to normal control fish

Meiogynogens		Controls		Place	Reference
% ♀	% ♂	% ♀	% ♂		
61	39	80	20	Pellestrina (Italy)	Barbaro *et al.*, 1997
64	36	53	47	Monfalcone (Italy)	Colombo *et al.*, 1996
70	30	48	52		
82	18	22	78	Eilat (Israel)	Gorshkova *et al.*, 1996

(viii) Complete sexual inversion of either sex by precocious administration of heterologous sex hormones with the diet can be easily accomplished in seabass, similarly to other teleosts (Pandian and Sheela, 1995). Complete masculinization by dietary androgen of female seabass was firstly reported by Blázquez *et al.* (1995). Feeding 17α-methyltestosterone at the dose of 10 mg·kg^{-1} food during 100 or 200 days starting 126-226 days post-hatching increased the percentage of males from 79% in the controls to 100%. Treatments started after 226 days post-hatching were ineffective. The same androgen dose given earlier (65- and 95-day-old fish) and for shorter periods (75 and 100 days) also resulted in 100% males (Gorshkova *et al.*, 1996). Further, masculinization was equally effective in 90-day-old fish at same dose applied for only 30 days or at a 10-fold lower dose (1 mg androgen·kg^{-1} food) for 60 days. At 23 months of age, pseudomales were fluent with milt, indicating a functional sexual inversion (Colombo *et al.*, 1996). Given the great sensitivity of seabass to androgen, treatment may be probably attenuated even more.

This species is also responsive to estrogen administration because a high dose of estradiol (125 mg·kg^{-1} food) given to 65- and 95-day-old fish for 75-100 days yielded 100% females. In this case too, it is worth exploring lower dosages and shorter periods of treatment.

All-female stocks of seabass would be certainly more productive in culture, as females grow faster than males and mature sexually 1-2 years later (Carrillo *et al.*, 1995). Nonetheless, if, unlike in trout farming, all-female progenies cannot be obtained by crossing normal females with pseudomales due to the problems discussed at point 7, then direct feminization might be an alternative to be

considered. In the European Community, this would require the addition of a specific authorization in the existing Directive (Council Directive 96/22/EC of 29 April 1996). This forbids the administration to aquaculture animals of substances with androgenic or estrogenic action (Art. 3) but authorizes the treatment of young fish for the first 3 months with allowed androgen under veterinarian control for the purpose of sex inversion (Art. 5). The extension of this exception to natural estrogens to feminize seabass appear not to be objectionable on a scientific basis under specific circumstances, such as:

(a) Inclusion in the feed of fish ovarian meal, like ground dried trout ovaries which are a waste product of the filleting process but are enriched in estrogen at maturity; alternatively, preparation of an equivalent feed obtained by the direct addition of estradiol-17β in a similarly low concentration; and

(b) Administration of such feeds to seabass just after weaning and for less than three months, especially if raised in a closed system with recirculating water; otherwise, the water effluent may undergo a depuration step (biological filtration, lagooning, etc.) to cut down any residual estrogen leakage into the environment. This approach would be absolutely safe for the consumer because no measurable trace whatsoever of exogenous estrogen would be left in the meat after more than one year in culture. It is noteworthy that estrogen production in fish is more widespread than in mammals, as substantial quantities are produced also by the brain and other organs, besides the ovary (Belvedere *et al.*, 1997). This normal endogenous concentrations would overshadow any improbable remnant of feminizing estrogen.

(ix) Hormonal sterilization has not yet been carried out in seabass, but partial sterilization was reported when masculinizing or feminizing treatments were performed in older fish, i.e., at 115 and 145 days of age (Gorshkova *et al.*, 1996). Complete sterilization is likely to require prolonged hormonal exposure, as in other species, but this engenders problems about consumer safety and acceptance, environmental protection against hormonal residues, and producer's convenience because seabass growth was found to be adversely affected by feeding androgen for more than 100 days (Blázquez *et al.*, 1995). Therefore, at present, this approach seems impractical.

Interspecific and intergeneric hybridization

Interspecific and intergeneric hybridization has contributed greatly to genetic and productive improvements in agriculture but much less so in terrestrial animal husbandry, which has mostly privileged interracial (intraspecific) hybridization. In fish culture, however, interbreeding of different species or genera may be of greater practical value than generally believed, especially in marine fish farming. Facilitated by their prevalently external mode of reproduction, high fecundity, frequent mass spawning habits, and rather common reciprocal genetic compatibility, teleosts can hybridize spontaneously in nature and are relatively easy to hybridize in culture. Actually, hybrids have been produced within each bony fish family that includes species breeding in captivity. Several excellent reviews have been published on this topic (Hubbs, 1955; Chevassus, 1979; Tiews, 1987; Campton, 1987; Purdom, 1993a; von Lukowicz *et al.*, 1990; Wohlfarth, 1994). Exploitability in culture of

hybridization within the families Moronidae and Sparidae has been recently discussed by Colombo *et al.* (1996).

Natural hybridization

Closely related species with isochronous reproductive seasons, and especially those relying on communal spawning rather than on complex courtship patterns, are more likely to hybridize in nature. Spontaneous hybrids, however, are relatively rare among marine species as compared to those living in freshwater. Here, environmental instability precipitating confinement of different species within a restricted water body, reduction of spawning grounds by man-induced habitat alterations, massive restocking with kindred species or introductions of alien species are often cited as causes of hybridization in fishes. The lesser importance of these factors in the marine environment and its relative stability may explain the rarity of marine fish hybrids (Campton, 1987; Purdom, 1993a).

Among freshwater teleosts, intergeneric hybrids are common in the family Cyprinidae, whereas hybrids tend to be intrageneric in the other families. Normally, only F_1 hybrids of wild spawners are found in nature, sometimes as reciprocal hybrids. Introgressive hybridization by backcrossing and multiple hybridization occur exceptionally because of hybrid sterility, reduced fertility or inadequate mating ability. They are nevertheless reported, as the finding of a natural triple bass hybrid resulting from the crossing between a hybrid (probably *M. chrysops* x *M. saxatilis*) and a third species, the yellow bass, *Morone mississipiensis,* in Galvestone Bay, Texas (Ward *et al.*, 1995). Sometimes, as in salmonids, F_2 hybrids are less fit than F_1 hybrids (Purdom, 1993a).

Artificial hybridization

In culture, straight F_1 hybridization can be carried out by: (i) volitional tank spawning of captive females of one species held in cohabitation with males of another species, particularly if gamete release is stimulated by exogenous hormones; (ii) artificial fertilization of the eggs of one species with the fresh sperm from a different reproductively synchronous species, or viceversa to generate the reciprocal hybrids; and (iii) artificial fertilization of the eggs of one species with the cryopreserved sperm from another species, thus multiplying the number of possible crosses by inclusion of males belonging to nonsynchronous or exotic species.

Whenever F_1 hybrids are at least partially fertile, there are five conceivable options for further hybridization, such as: (i) breeding of equivalent hybrids from the same parental species (F_2 hybridization); (ii) intercrossing of reciprocal hybrids from the same parental species (F_2 reciprocal hybridization); (iii) intercrossing of hybrids from different parental species, with four possible combinations of hybrids and their reciprocal hybrids (double hybridization); (iv) backcrossing of hybrids with one or both of their parental species (introgressive hybridization); and (v) crossing of hybrids with a third non-parental species (rehybridization).

Hybridization and selection

In all these cases, owing to the casual segregation of the parental chromosomes during gametogenesis in the F_1 hybrids, the genetic contributions of each original parental species (F_0) to the diploid chromosome set of each new F_2 hybrid will be totally at random. This genotypic heterogeneity among F_2 hybrids is likely to be associated with phenotypic heterogeneity in terms of performance in culture. Apart from introgressive hybridization that, through successive crosses, tends to disperse the initial shuffling of heterospecific chromosomes within the broader genetic pool of the parental species, thus minimizing genotypic heterogeneity, the latter will be perpetuated when applying F_n hybridization or augmented with double hybridization or rehybridization. The picture may be further complicated by crossovers between parental chromosomes, if present. This means that selection of F_n hybrids for complex traits will be much more difficult to control than in pure species because of the excess of variation, unless backcrossing is introduced at an early stage. As reported by Purdom (1993a), the introgressive approach was successfully adopted in the Soviet Union where the intergeneric hybrid of the common carp x crucian carp, *Carassius carassius*, was backcrossed to confer upon one parental species distinctive traits of the other parental species. Hybrids between *Oncorhynchus mykiss* and brown trout produced some trout of golden appearance; but the hybrid, as expected, did not breed true (Purdom 1993a).

Reports of this sort are sporadic and scarcely documented but they are of interest being examples of (multiple) gene transfer by conventional breeding techniques. The fact that foreign genes were inserted into a species by genetic intermingling through hybridization rather than by genetic addition through egg microinjection or gamete electroporation does not seem to justify, in such trials with fertile fish, the total lack of those containment measures that are usually imposed on experiments with transgenic fish.

The use of interspecific hybridization as a preliminary step to selective breeding appears to be of questionable practical advantage because of the frequent low fertility of hybrids, the long period of time required for the introgression of the heterospecific trait by repeated backcrossing, the risk of the accidental dispersal of the fertile hybrids or their introgressed descendants into the natural environment during the handling of the numerous fish under selection, and the possibility of losing the heterospecific trait during the course of introgression. Rather than transferring genes as a result of hybridization followed by selective introgression, it looks much more convenient to postpone hybridization to gene transfer by gamete or embryo manipulation in order to exploit hybrid sterility for the reproductive containment of the transgenic fish, as suggested below.

Hybridization and chromosome set manipulation

Another attractive approach is the integration of interspecific and intergeneric hybridization with chromosome set manipulation techniques, as outlined above. Allotriploid fish obtained either by hybridizing two diploid spawners belonging to different species with subsequent retention of the second polar body, or by crossing allotetraploids with diploid mates, should be absolutely sterile because of both their

hybrid nature and triploid condition. Allotriploids produced by crossing female *S. aurata* x male *P. major*, with second polar body retention, could be differentiated phenotypically from diploid hybrids, even at the fingerling stage, thus making a check on ploidy status directly feasible (Gorshkova *et al.*, 1995).

Induction of triploidy may be applied to hybrids from taxonomically related species in order to eliminate any residual fertility and risk of introgression in nature with the parental species. This risk is often neglected in fish culture on the grounds that, even if fertile, hybrids are not inclined to reproduce in the wild, as indicated above. Such an argument may be further strengthened whenever equivalent or similar hybrids arise sometimes spontaneously in nature due to weak interspecific boundaries. This is probably the case of the striped bass hybrids of North America, as discussed below.

Triploidy may also be the natural outcome of a hybridization event. Females of the reciprocal crosses *O. latipes* x *Oryzias curvinotus* show reduced fertility and ovulate diploid eggs with one complete chromosome set for each parental species so that, when backcrossed, they give rise to a triploid progeny (Kurita *et al.*, 1995). A similar finding was reported also for the hybrids of the cross crucian carp, *C. auratus gibelio* x common carp (Cherfas *et al.*, 1994).

In comparison with proximal hybrids, sterility is expected to be more severe or complete in diploid hybrids born from systematically distant species, like in intergeneric hybridization, owing to unsuccessful pairing of the homologous chromosomes (Stoumboudi and Abraham, 1996). As a consequence, gonadal development is often markedly impaired.

Partial genetic hybridization achieved by both fragment gynogenesis and nuclear-cytoplasmic hybridization by either androgenesis or nuclear transplantation is of great experimental interest, but it poses containment problems for farming because this kind of artificial hybrids is not expected to be sterile. A technique has been developed in the People's Republic of China for the production of nuclear-cytoplasmic hybrids by transplanting nuclei from common carp eggs into the cytoplasm of *C. auratus* eggs. It was shown that the expression of meristic traits, such as the number of vertebrae or pharyngeal teeth, is influenced by both the nucleus and the cytoplasm (Research Group of Cytogenetics *et al.*, 1981; Lu *et al.*, 1993).

Advantages of hybridization

Besides sterility, hybrids may display other positive traits that nevertheless cannot be predicted from the knowledge about their parental species and may be absent altogether, particularly in certain teleost families. According to Purdom (1993a), very few of the interspecific and intergeneric hybrids of salmonids and cyprinids have any farming or restocking potential because of their low viability or inferior performance with respect to the parental species.

When things go otherwise, there are five possible outcomes of hybridization which may result beneficial: intermediacy, combinability, luxuriance, monosexuality and novelty.

Intermediacy, that is the fact that morphometric and meristic traits of the hybrids are somehow intermediate between those of the parental species, has been confirmed in several cases (cf., Hubbs, 1955). This may create more appealing forms of fish, particularly in ornamental fish culture.

Combinability refers to the possibility of combining in the hybrids favourable characteristics proper of each parental species. For instance, the sturgeon hybrid, called the bester, combines the fast growth rate of the great beluga, *Huso huso*, with the freshwater tolerance of the tiny sterlet, *Acipenser ruthenus* (Shiraishi *et al.*, 1993). As the bester is a fertile, self-perpetuating breed, it provides an example of artificial speciation tolerated because of its benefits.

Luxuriance is an important goal of artificial hybridization and is mostly meant to denote a superior growth rate of the hybrids with respect to the parental species. Enhancement of at least early growth rate is a recurrent feature in several hybrids and may reflect the attenuation of inhibitory regulations on growth whenever the underlying molecular mechanisms inherited from the parental species result incompatible or poorly harmonizable within the hybrid.

For instance, the reduction of the energy expenditure for defence metabolism, especially that involved in the reaction to stress stimuli, may divert more energy towards auxogenic processes (Colombo *et al.*, 1990). Interestingly, the sunshine bass (female *M. chrysops* x male *M. saxatilis*) shows a lower responsiveness to stressors than the paternal species, as measured by the increase in plasma cortisol level induced by a standardized confinement stress (Noga *et al.*, 1994). This trait, that would be hazardous in nature since it reduces alertness towards threats, becomes a positive attribute in culture because it leaves more energy for a faster early growth rate and increases resistance to infection, and hence survival, as a consequence of lighter corticosteroid immunosuppression.

Luxuriance should not be confused with heterosis, i.e., the improvement of performance that marks certain crosses between conspecific inbred lines. Luxuriance is the outcome of hybridization *per se*, that is the mixing of genomes belonging to distinct phylogenetic lines, and is likely to be more evident in wider taxonomic crosses, while being relatively independent from the particular genotypes of the spawners. The superiority of the luxuriant hybrids with respect to both parental species is more easily explainable as a subtractive effect, namely the loss of an unwanted function while retaining all other vital capacities and homeostatic power, hence a fortunate compromise, rather than as a synergistic complementarity between parental traits. This luxuriance event is more likely to occur when the parental species have genomes that are equivalent in size and chromosome number. Otherwise, aneuploidy and developmental abnormalities may ensue (Gui *et al.*, 1993).

If F_1 hybrids are fertile, luxuriance may not be maintained in F_2 hybrids because of the aforementioned random recombination of the parental chromosomes, and because crossovers, if any, between parental chromosomes in the F_1 generation will cause chromosomal hybridization, i.e., exchange and incorporation of heterospecific chromosomal segments, with possible detrimental positional effects among genes linked within the same chromosomes. This would be in agreement with the observed inferior fitness of salmonid hybrids in the F_2 progeny than in the F_1 (Purdom, 1993a).

Conversely, heterosis is not due to interbreeding by itself but depends upon the particular genotypes of the inbred lines to be crossed. These must give rise to a propitious heterozygous condition in which specific genetic determinants promote positive effects through dominance or overdominance interactions. Heterosis too is not maintained in the F_2 and subsequent generations owing to the decrement of heterozygosity in part of the offsprings. So, both luxuriance and heterosis represent short-term genetic gains, in the sense that they are attainable in the first progeny and cannot be improved further. Their immediate appearance, however, does not mean that they can be achieved quickly because to find the right combination of species for luxuriance or inbred lines for heterosis may take quite some time.

Monosexuality is sometimes exhibited by interspecific hybrids and may be exploited whenever the exclusive sex performs better in culture than the other. All-female hybrids were produced from the cross between female *M. saxatilis* x male *M. mississippiensis* (Wolters and Demay, 1996). All-male hybrids can be obtained by crossing different tilapia species but their use on a large scale has not been entirely successful, as females reappear in later progenies because of broodstock infiltration by hybrids. Thus, tilapia hybridization has been largely replaced by direct hormonal masculinization (Wohlfarth, 1994). The use of androgenetic YY males to produce all or nearly all male (XY) progeny in crosses with normal XX females has been also proposed as a valid alternative in the Nile tilapia (Mair *et al.*, 1995).

Novelty consists in the appearance in the hybrid of a positive trait that was not present in the parental species. An example is provided by the appealing marbled colour pattern of the so-called "tiger" trout, the hybrid of the cross between female brown trout (*Salmo trutta*) x male brook trout (*Salvelinus fontinalis*), that was superior in culture to its reciprocal and triploid variants (McKay *et al.*, 1992).

Interspecific and intergeneric hybridization in marine fish culture

Hybridization experiments in marine teleosts are still limited in number as compared to freshwater species but indicate that, especially in the families Moronidae and Sparidae, hybrids may have a considerable potential for commercial culture.

Hybridization in the family Moronidae

Among Moronidae, a growing literature exists on striped bass hybrids in North America. The striped bass is a euryhaline anadromous species, while the white bass, *M. chrysops,* is a freshwater relative. The original cross, female striped bass x male white bass, was first performed in South Carolina in the mid-1960s and the resulting hybrid was named palmetto bass. Its reciprocal hybrid, the sunshine bass, became even more popular.

As compared to striped bass, these hybrids are appreciated for their faster early growth, good osmoregulatory capacity and thermal tolerance, greater resistance to stress and infections (Noga *et al.*, 1994), higher survival in reservoirs and different culture units (ponds, cages and indoor tanks), propensity to predate and control unwanted fish species (Detimers *et al.*, 1996), and susceptibility to utilize soybean

meal as a source of protein (Gallagher, 1994), while consumer acceptance is similar to that of striped bass (Hallerman, 1994).

Hatchery production of hybrids relies at present on artificial fertilization of gametes stripped from spawners induced to maturity with implanted delivery systems containing a gonadotropin releasing hormone analogue (GnRHa) (Mylonas *et al.*, 1996). Volitional tank spawning of female striped bass with male white bass has been also reported after GnRHa treatment, but yield of fertilized eggs was low owing to some behavioural aversion of the females to spawn with heterospecific males (Woods *et al.*, 1995b).

Several other hybrids have been produced, including striped bass x white perch (Noga *et al.*, 1994) and striped bass x yellow bass hybrids (Wolters and Demay, 1996). The latter hybrids were all-female but grew less than palmetto bass. The cross white bass x yellow bass was reported to occur spontaneously in reservoirs in Texas (Fries and Harvey, 1989).

Bass hybrids may show some degree of fertility (Leclerc *et al.*, 1996), but evidence of introgression or F_2 hybridization in the wild is still inconclusive (Purdom, 1993a; Woods *et al.*, 1995a), though a case of spontaneous rehybridization has been documented, as indicated above (Ward *et al.*, 1995). Despite their fertility and phenotypic superiority, restocked or escaped bass hybrids do not seem to represent a greater threat to the genetic integrity of the pure bass species than the naturally bred hybrids. So far, no hybridization experiment has been conducted in Mediterranean moronids, like the seabass.

Hybridization in the family Sparidae

While hybridization experiments in the family Moronidae have been performed within the same genus, in the family Sparidae attention has been focused on intergeneric hybrids. The first report on intergeneric hybridization of Mediterranean sparids was published by Dujakovic and Glamuzina (1990) who obtained viable offsprings reared up to 30 days post-hatching from the intercrosses female gilthead seabream x male sharp-snout seabream (*Diplodus puntazzo*), and female gilthead seabream x male two-banded seabream (*Diplodus vulgaris*). Both these hybrids and another hybrid from the cross female white seabream (*Diplodus sargus*) x male common dentex (*Dentex dentex*) (Dujakovic and Glamuzina, 1993) showed hatching rates and growth rates up to day 30 not significantly different from those of the maternal species. The cross female red seabream x male black seabream (*Acanthopagrus schlegeli*) produced hybrids with excellent hatching rate which could be grown to adulthood (Sugama *et al.*, 1990b). Hybrids of female gilthead seabream x male red seabream were bred in Israel and found to be entirely sterile for lack of gonadal development up to 3 years of age (Diskin, 1993). Sterility was confirmed in the same hybrids, obtained in both diploid and triploid conditions, by Gorshkova *et al.* (1995).

In 1990, at the marine fish hatchery of Maricoltura Italia SpA, Monfalcone, Italy, research on intergeneric hybridization of sparids was triggered by a serendipitous finding. A batch of fertilized eggs collected from a spawning tank holding common dentex broodstock was found to give rise to fry with a marked bi-modal growth curve

as one group of fingerlings was growing at a much faster rate than the rest. Morphologically, these fingerlings were similar but not identical to normal dentex. Genetic analysis showed that they were actually hybrids of female common dentex x male red seabream. A subsequent search through the hatchery's register confirmed that a male red seabream had indeed been spotted and removed from the spawning tank of common dentex during cleaning operations. This single male was, therefore, capable of quite successful volitional spawning with common dentex female(s). At 4 years of age, this kind of hybrids, nicknamed dentagrus after the parental genera *Dentex* and *Pagrus*, was found to be sterile for lack of gonadal development beyond a vestigial stage.

Given the remarkable growth rate of dentagrus, the reciprocal hybrids, named pantex, were produced by artificial fertilization since 1991. This particular type of cross was preferred to the original one because it was much easier to obtain good quality eggs from females of red seabream than common dentex. Pantex displayed immediately an impressive growth potential which, in a monitored trial, averaged 46 g at 139 days post-fertilization. After ongrowing at the hatchery in water warmed by the effluents of a power station, pantex reached 1-1.2 kg at 2 years of age and were characterized by a lack of gonadal development (Colombo *et al.*, 1996). When raised in floating cages at sea, performance was again very good (1.4 kg at 2 years) in the relatively warm waters of Greece, but it was disappointing in the North Adriatic. This evidence of a thermal preference suggests that pantex is fit for cage culture in the southern part of the Mediterranean Sea. On the market, pantex were sold at a favourable price with good consumer acceptance, as judged from retailers' comments.

Other hybridization experiments carried out at the same hatchery included the cross female red seabream x male gilthead seabream, named pagrarus, the reciprocal hybrid of that reported by Diskin (1993) and Gorshkova *et al.* (1995) in Israel, and the cross female gilthead seabream x male common dentex, named sparantex. Both hybrids were fully viable and had a good early growth (41 g at 200 days post-fertilization for pagrarus), though inferior to that of pantex (Colombo *et al.*, 1996).

Intergeneric hybrids of sparids appear to be good candidates for mariculture because of the following traits: (i) good growth performance, in some cases superior to those of the parental species; (ii) gonadal sterility, ensuring reproductive isolation and prevention of genetic threats to wild fish; and (iii) good viability and appealing morphology of the hybrid progenies derived from an array of different crosses, so that production can be diversified at a low cost to meet market demands and to avoid market saturation.

The main disadvantages of rearing sparid hybrids instead of the pure species are: (i) the need to fertilize eggs artificially, since volitional spawning is less likely to occur in heterospecific encounters; many sparids have asynchronous ovaries with multiple ovulatory cycles and the stress stimuli caused by capture and egg stripping may terminate their ovulatory cycling; and (ii) since hybrids are genetic dead ends, their performance cannot be improved further by selective breeding, though this can be done with their parental species.

For the latter problem, however, there is a neat solution. In fact, fast-growing sterile hybrids would be ideal recipients for gene transfer to enhance growth further or to confer any other positive attributes. There are two reasons for this: (i) compliance with the regulations about dependable biological containment for any transgenic fish, due to sterility; and (ii) the fact that the introduction of exogenous genes into hybrid genomes, that actually combine two sets of mutually foreign genes, is not as objectionable as in the case of natural or domesticated pure genomes. Trangenesis would merely accentuate the artificial nature of the hybrids. Whether this may be also considered a valid line of reasoning to support patenting is debatable because hybridization cannot be patented *per se* (as it may occasionally occur spontaneously) and, thus, the legal position of hybrid transgenics would be equivalent to that of non-hybrid transgenics.

At this point, an innovative breeding scheme can be envisaged in which a limited number of transgenic females of one species are raised under strict and controlled physical confinement in order to provide eggs for fertilization with sperm of a different compatible species. The resulting transgenic hybrids, bearing the transgene in a hemizygous condition, would then be cultured to market size. Of course, the scheme may be also reversed by hybridizing transgenic males with normal females. This approach has two merits: (i) it reduces to a minimum the number of transgenic broodfish necessary to generate a variety of fish for farming, thus facilitating supervision and control by appointed public agencies; and (ii) it allows market control by the enterprise owning the transgenic broodfish.

Presently, research is under way to better characterize pantex and other sparid hybrids morphometrically, to evaluate their metabolic capacity and to confirm their total sterility in order to assess their suitability for transgenesis.

Hybridization in the family Sciaenidae

Prospects for culture similar to those outlined in the family Sparidae may also exist in other teleost families. Among Sciaenidae, the hybrids from the cross female black drum (*Pogonias cromis*) and male red drum (*Sciaenops ocellatus*) were found to grow faster in saltwater ponds than the parental species without any difference in flavour (Henderson-Arzapalo *et al.*, 1994). Recently, viable hybrids have been obtained also from the cross female corb (*Umbrina cirrosa*) x male brown meagre (*Sciaena umbra*) (Barbaro A. and Francescon A., unpublished).

Genetic engineering

Following the pioneering experiment by Zhu *et al.* (1985) concerning the transfer of a growth hormone (GH)-encoding genetic construct into the fertilized eggs of the goldfish to enhance body growth, a vast body of literature has accumulated on the topic of transgenesis in teleost fish. Several extensive reviews have monitored progress in this field and only a list of the most recent ones is provided here (Powers *et al.*, 1992; McEvoy *et al.*, 1992; Jiang, 1993; Maclean and Rahman, 1994; Chen *et al.*, 1995; Gong and Hew, 1995; Yiengar *et al.*, 1996; Knibb *et al.*, 1996; Knibb, 1997). In broad terms, gene transfer in fish is exploited in both basic and applied research.

Fish transgenesis in basic research

In fundamental science, gene transfer in fish is a valuable tool in the study of the regulation of gene expression, particularly in the areas of developmental biology and evolution of functional genomics, and relies chiefly on two aquarium fishes as experimental models, namely the zebrafish and the medaka.

The main advantages of these fish (and other similar model fishes, such as platyfish and swordtails) for gene transfer as compared to mammalian systems, like transgenic mice, are: (i) the large and continuous availability of gametes and embryos for direct manipulation at a low cost; (ii) the relative rapidity of embryonic development; and (iii) the transparency of embryos, prelarvae and larvae (up to the pigmentation stage) for direct localization of reporter gene transcription. These features make these fish very handy especially for *in vivo* transient gene expression assays.

On the other hand, there are also some disadvantages with piscine systems that still need be overcome since they lower considerably the transgene integration efficiency and hinder the production of stable transgenic lines and transgene targeting within the recipient genome. The first obstacle is the yolk content of fertilized eggs that prevents pronuclear microinjection, as it is routinely done in the mouse. Hence, transgenes must be introduced into the ooplasmic compartment of postovulatory oocytes and fertilized eggs or into the blastomeric cytoplasm of early-segmenting embryos using a variety of techniques (microinjection through the chorion or micropyle, electroporation, particle gun bombardment, and transfection with liposomes). This approach requires the use of a much higher number of transgene copies (10^5-10^7 copies/cell instead of 200 copies/pronucleus, as in the mouse) in order to favour nuclear incorporation and chromosomal integration. Despite this compensation, transgene integration tends to be retarded and its efficiency rather low in fish (Knibb *et al.*, 1996; Iyengar *et al.*, 1996).

At present, medaka is the only teleost in which foreign DNA can be successfully injected into the polarized germinal vesicle of primary oocytes, but this techniques involves complex procedures and still requires $5x10^3$-10^4 transgene copies/nucleus (Ozato *et al.*, 1992). Effectiveness of gene transfer mediated by sperm cells subjected to incubation or electroporation with the exogenous DNA, though claimed in zebrafish (Khoo *et al.*, 1992), seems to be very low or negligible (Chourrout and Perrot, 1992; Müller *et al.*, 1992; Knibb *et al.*, 1996), as observed also in mammals (Niemann, 1996).

The second obstacle is the extremely rapid succession of the first 10-11 cleavages in fish embryos without G_1 and G_2 phases and virtually no transcription up to the mid-blastula stage. This fast segmentation is presumably supported by a large store of proteins and precursors for DNA replication and chromatin assembly that is not available in mammals, whose early development is much slower (Iyengar *et al.*, 1996).

This state of affairs tends to promote: (i) replication of incorporated DNA, mostly in an extrachromosomal state, with an overall increase in the number of copies/nucleus (Volckaert *et al.*, 1994b); (ii) concatemerization (i.e., formation of tandem arrays) of

incorporated and replicated exogenous DNA sequences carried out by stored ligases, whose activity is likely stimulated by their high concentrations (Iyengar and Maclean, 1995); (iii) mosaicism of expression and chromosomal integration, if any, of each single transgene within the embryo as a result of unequal initial nuclear incorporation and differential replication, degradation or subsequent fate of transgene copies (Williams *et al.*, 1996); (iv) transgene silencing by DNA methylation and heterochromatin formation that seems to be more intense when integration occurs as multiple-copy concatamers and is probably responsible for the fading away of transgene expression in successive fish generations (Gibbs *et al.*, 1994).

These drawbacks prevent the non-mosaic integration of the transgene as a single intact copy as opposed to concatamers. Moreover, they hinder the application of two-step transfection techniques, such as the use of cultured pluripotent embryonal stem (ES) cells as foreign DNA vectors in mice, to perform transgene targeting, like gene knockout and gene replacement through homologous gene recombination (Suemori *et al.*, 1990). Although ES cell lines can be derived from blastula-stage embryos of zebrafish and grown in culture, where they can be transfected and selected for the desired copy number, expression level and genomic localization of the transgene (Collodi *et al.*, 1992; Sun *et al.*, 1995), their transplantation into pre-mid-blastula stage fish embryos to give rise to chimeric transgenic somatic tissues and germline is not as straightforward as in mice. In fact, the proliferation rate of transplanted ES cells is much slower than that of the cells in the recipient embryo, probably because they are not endowed with the same aforementioned replicative machinery, and their competition to contribute to the total cells of the embryo is consequently curtailed. Since later on only a small number of gametes will be transgenic, if any, the production and identification of transgenic progeny becomes cumbersome.

Alternative approaches are worth exploring to improve the control on gene transfer in fish to a level comparable to the much more successful research with transgenic mice. A promising avenue would be the transplantation of single decondensed sperm nuclei, transfected by restriction enzyme-mediated integration of the transgene, into unfertilized eggs. This technique has been fruitfully applied in *Xenopus laevis* to produce embryos expressing the transgene integrated in a few copy number (as single copy or short (2-6 copy) concatamers), nonmosaically and with good tissue-specificity or regional restriction (Kroll and Amaya, 1996).

This method yields a transgenic progeny with the transgene integrated in a hemizygous condition at different genomic sites that can be upgraded to a homozygous condition, at least at a single integration site, in part of the subsequent offspring obtained by normal reproduction. This process can be speeded up by resorting to reproduction by mitogynogenesis or diploid androgenesis instead of biparental breeding so to generate at once completely homozygous transgenic animals serving as progenitors of clonal stabilized transgenic lines.

A further conceivable extension of the nuclear transplantation technology would be the microinjection of single nuclei from *in vitro* transfected and selected ES cell into genetically inactivated eggs to produce non-chimeric transgenic fish bearing targeted transgenes. Homozygosity of transgenes could then be achieved later on by unisexual or bisexual reproduction. These developments, if implemented, would

eliminate the present shortcomings of ES cell technology in fish and merge eventually into a technology for the transfer of nuclei from a variety of somatic cell types stably transfected in culture. The latter approach was used to produce transgenic sheep, since in this species ES cells capable of contributing to the germline have not yet been isolated (Schnieke *et al.*, 1997).

Gene transfer to improve production in fish culture, especially in the Mediterranean region

Transgenesis aimed to benefit fish culture has been mainly performed in freshwater-spawning teleosts, that is cyprinids, salmonids, cichlids, esocids and ictalurids (cf., above reviews). Most experiments have concentrated on growth enhancement by microinjection of GH-encoding genetic constructs into fertilized eggs, but research has also been directed towards increasing the freeze resistance of Atlantic salmon (Fletcher *et al.*, 1992) and cold tolerance of goldsfish (Wang *et al.*, 1995) as well as to develop a transgenic approach to confer disease resistance (Jiang, 1993; Anderson *et al.*, 1996).

In addition to the difficulties and problems discussed above regarding gene transfer in fish in general, three other issues need be considered when dealing with transgenic fish for massive culture, namely environmental safety, economic convenience and consumer acceptance.

Environmental safety

The problem of safety has been repeatedly raised (Kapuscinski and Hallermann, 1991; Hallerman and Kapuscinski, 1992; Hindar, 1993) and can be best overcome by rearing a limited number of transgenic broodfish in closed systems with recirculating water to ensure complete physical confinement, and by growing for fattening only totally sterile fish produced by interspecific hybridization, triploidy induction, genetic sterility induced by homozygous recessive alleles (Knibb *et al.*, 1996), expression of a GnRH anti-mRNA driven by a GnRH gene promoter (Alestrom *et al.*, 1992) or other equivalent means. Risk assessment studies, based on theoretical inference or even pilot release trials, in order to figure out the possible undesirable environmental impacts of fertile transgenic fish set loose into the wild, have uncertain predictive value and applicability, as amply discussed by Hindar (1993), and may actually cause dispersal of transgenic fish.

Nevertheless, Knibb (1997) takes a strong stand against the assumption that the release of genetically modified fish, including warmwater marine fish, like the gilthead seabream, that have a great potential for dispersal, a low probability for recapture and a reasonable chance to survive and reproduce in the wild, may in any way represent an implant of superior variants capable of genetic amplification at the expense of feral conspecifics and other aquatic populations. On the opposite, based on the previous ecologically risk-free record of genetically engineered domestic plants and terrestrial animals, he contends the hypothesis that laboratory-induced genetic changes "have a negligible probability of being selectively favoured in wild populations under natural selection, and accordingly, without sustained large releases, have little potential for ecological impact". The crucial argument is that

without substantial knowledge of the complexities in genomic architecture and gene interplay required for phenotypic adaptation to the environment, any engineered genetic change is fated to exalt a peculiar trait at the detriment of overall fitness in the wild, in a similar way as classical random mutations.

This reasoning is well substantiated by existing evidence and may be endorsed to remove excessive concern about controlled research on gene transfer in fish and the setting up of transgenic broodstocks of a limited size under physical confinement. This policy would be analogous to the official guidelines for gene transfer in the mouse, a cosmopolitan pest species with thousands of transgenics already produced, none of which has consolidated in the wild.

On the other hand, reliance on a precautionary attitude towards the mass cultivation near or at the sea of non-sterile transgenic marine fish is likely to prevail at present, justified by lack of pertinent data and our scarce control over the marine environment. Restrictive regulations will then continue to be enforced, unless one of the following conditions are met: (i) conclusive proof that non-sterile transgenic fish cannot propagate in the wild; (ii) demonstration of the complete and permanent sterility of transgenic fish for ongrowing; or (iii) fattening of marine transgenic fish in farms operating with recirculating systems in the hinterland, away from the sea, in the case that the previous two conditions have not been convincingly fulfilled.

Economic convenience

As to economic convenience, the question of whether it might be profitable for the producers in the Mediterranean region to invest in transgenic technology can be answered at this time only in general terms owing to the fact that interest in this opportunity is merely incipient. Yet, a number of possible advantages can be easily put on a list, such as: (i) improvement of disease resistance in transgenics would be a valuable trait, considering the economic losses caused by recurrent epizootics in marine fish culture; (ii) enhancement of growth at the larval and perimetamorphic stages may reduce the considerable costs of the current rearing technology, based on the prolonged administration of living preys, by anticipating the time of weaning with an inert diet; (iii) amplification of anabolism in fast-growing transgenics may ameliorate the conversion efficiency of high energy feed, like extruded pellets with a high lipid content, into a lean filet meat, thus reducing unwanted accumulation of intramuscular and perivisceral fat; an increase in food conversion efficiency (up to 30%) by administered recombinant fish GHs has been repeatedly reported (cf., Knibb *et al.*, 1996) (iv) fast-growing transgenics are best suited for culture in recirculating systems where the benefits of optimized culture conditions, protection from pathogens and negligible polluting discharges are counteracted by high running costs that can be sustained only if the production cycle is considerably shortened; (v) genetic engineering of cultured carnivorous species to confer additional digestive and metabolic capabilities (euryphagy) may allow feeding on less expensive nutrients, such as carbohydrates or vegetable oils (Gong and Hew, 1995; Knibb *et al.*, 1996), thus partially replacing animal protein and fish oil in the diet; (vi) the ownership of a transgenic broodstock is likely to afford a control over a share of the fish market that is expected to grow larger and larger, the lower the price of the marketed transgenic fish; and (vii) expertise gained in the transgenic culture of Mediterranean fish species may be put to use in co-operative programs with

developing countries centred on other species, such as cyprinids and cichlids, that are important for a multitude of consumers with a limited supply of animal protein.

Consumer acceptance

With regard to consumer acceptance, this is likely to evolve together with the public perception of genetically modified organisms as a result of the combined conflicting influence of traditional values, ethical issues, ideological positions, political mood, mass media orientation in information processing and science popularization and other unpredictable variables. Since there is no need for a transgenic technology in marine fish culture unless it can supply a product of superior quality at a lower price, a transition in consumers' attitude towards transgenic fish can then be envisioned, on the basis of sheer convenience, from initial refusal to tolerance and, finally, to appreciation.

A similar trajectory can be recognized in the course of the last twenty-five years with respect to cultured vs. captured seabass and seabream. With a drop in retail price to about one third of what was offered in the past, and the general good uniformity and dependable quality of the cultured product, few people really care nowadays whether the fish on sale had lived in a floating cage instead of struggling for life on the high seas. Actually, with a deepening perception of the existential value of wild life, it may be reassuring, if not comforting, to know that one's meal demanded the sacrifice of a creature born for that purpose and grown with care.

However, to believe that consumers' choices are motivated exclusively by a rational appraisal of sheer convenience is an oversimplification, as diffidence towards novelty is often a critical barrier to food product acceptance. This means that the marketing strategy for genetically engineered fish must concentrate not only on an appealing quality/price ratio but also on gradualness, moderation and fairness.

Gradualness consists in offering to consumers a series of intermediate products with increasing divergence with respect to consolidated items in order to soften the impact of novelty. A conceivable sequence may be: triploid and monosexual fish → sterile hybrids cultured in open or closed systems → diversified sterile hybrids of unimaternal/polypaternal origin → disease-resistant sterile transgenics → fast-growing sterile transgenics → double-muscled sterile transgenics (see below) → euryphagic sterile transgenics and so on. Of course, some of these novel traits could be engineered not only in parallel but also in series along the same transgenic line.

Presumably, only a part of the above sequence would be really implementable in Mediterranean cultured fish species, whose market volume is probably not big enough to justify huge research investments, if it were not for the fact that the same biotechnology may be profitably extended to other species of interest for third world countries.

Another point to take into account is that quantitative genetics is also being applied to fish in order to pursue some of the same goals as transgenetics, like disease resistance (Fjalestad *et al.*, 1993) and growth enhancement, (Knibb *et al.*, 1996). At present, it cannot be predicted what approach will meet with more success, whether the consolidated methodology of quantitative genetics with its onerous

logistics and gradual gains, or the technological dynamism of transgenetics with its present experimental diversification and search for sudden breaktroughs.

The requirement of moderation in cultured fish transgenesis refers to the recommendation of refraining from too radical genetic changes induced by transgenes with an overly artificial design. So far, all experiments have been aimed towards the direct phenotypic expression of gene constructs, whereas the possible benefits of knocking out the expression of constitutive genes by homologous recombination has not yet been investigated for lack of an adequate technology, as discussed above. In this respect, an attractive target might be the myostatin gene, if present in fish, whose inactivation by a random mutation - an 11-bp deletion - gave rise to the double-muscled Belgian Blue bovine strain (McKnight, 1997; Grobet *et al.*, 1997). Mice with a disrupted myostatin gene were significantly larger than wild-type animals and showed a 2-3 times increase in skeletal muscle mass (McPherron *et al.*, 1997). A transgenic fish with a better performance in culture because it bears a suppressed gene is likely to be more acceptable by the food market as compared to a fish actively expressing a transgene.

Most research on gene transfer to improve growth in farmed fish is now conducted with transgenes containing exclusively fish-derived sequences ("all-fish" constructs) in place of the mammalian, avian and viral regulatory and coding sequences used in early work. This positive trend towards a greater homology between the transgene and the recipient genome has not yet progressed further into the selection of more finely tuned promoters to drive the expression of complementary or genomic hormone-encoding DNAs. In fact, the pursuit of high expression levels of GH has prompted the use of chimeric constructs containing regulatory sequences with either a widespread tissue expression, such as the salmonid metallothionein gene promoters and the carp β-actin gene promoter, or with a tissue-specific expression of high intensity, such as the hepatotropic oceanpout antifreeze protein gene promoters. Unfortunately, the overexpression of GH is not always correlated with a better growth (Du *et al.*, 1992; de la Fuente *et al.*, 1997) and may bring about negative side effects.

Devlin and coworkers (1994) reported that coho salmon, *Oncorhynchus kisutch*, bearing a construct consisting of the sockeye salmon GH gene fused to the sockeye salmon metallothionein-B promoter, were on average 11-fold heavier (and some individuals up to 37-fold heavier!) than non-transgenic controls at 14 months of age and had GH plasma levels up to 40-fold higher due to an uncontrolled overexpression of the transgene. These fish were affected by various abnormalities, such as acromegaly, cranio-facial deformities and opercular overgrowth, and showed reduced viability. Recently, Rahman *et al.* (1997) have found that transgenic Nile tilapias, carrying a chinook salmon GH gene driven by an ocean pout antifreeze protein promoter, were characterized by a high level of exogenous GH expression and a 4 times greater growth rate than their non-transgenic full siblings. Transgenic males, however, were subfertile or completely sterile.

These and similar results suggest that a convenient alternative might be the transfer of GH gene constructs driven by their own regulatory sequences to target expression to the pituitary, because the increase of GH levels in the resulting transgenics would be more physiologically controllable. Oddly enough, gene transfer

experiments of this kind have never been performed so far in farmed fish, probably for the fear that internal negative feedback mechanisms might depress the expression of the transgene. Consequently, basic research on the transcriptional control of the GH gene in fish has not contributed at all to transgenesis applied for growth enhancement in fish culture.

Investigation on the functional organization of the promoter region of the GH2 gene of rainbow trout (rtGH2) as compared to those of mammals has revealed that the *cis*- and *trans*-regulatory elements governing GH expression are highly conserved in vertebrates and that they evolved essentially combinatorially (i.e., by different assortments and positioning of the same regulatory units within the various promoters) rather than molecularly (i.e., through the development of novel regulatory units) according to the contingent adaptive needs of each species (Argenton *et al.*, 1993). Another interesting aspect is that the proximal promoter region of rtGH2, stretching across about 200 bp 5' upstream of the transcription start, is sufficient to confer a strong tissue-specific expression to a reporter gene in transfection assays. In fact, this region contains 3 binding sites (F1-F3) for the conserved, pituitary-restricted transcription factor Pit-1, which is required for the expression in somatotropic cells (Argenton *et al.*, 1993; Andersen and Rosenfeld, 1994).

Moreover, the region encompassing the F2 and F3 Pit-1-binding sites includes two additional regulatory elements: a TGACG motif, corresponding to CRE (cAMP response element), that stimulates transcription upon interaction with the *trans*-factor CREBP (CRE-binding protein) once phosphorylated by protein kinase A; and a GRE-like (glucocorticoid response element) motif that activates transcription after binding with the saturated corticosteroid receptor. These two elements confer a synergistic responsiveness to cAMP and cortisol on the rtGH2 promoter. Interestingly, the promoter region located upstream of the F3 site is apparently inhibitory, even though it carries an additional Pit-1-binding site (F4) (Argenton *et al.*, 1996a).

These data suggest that the proximal promoter is essentially involved in transcription activation whereas other regulatory elements with either enhancer or silencer function may be located further upstream or elsewhere. Hence, the use of constitutive promoters of short length should ensure the eutopic expression of GH-encoding transgenes without responsiveness to internal negative feed-back mechanisms. Longer promoters may favour instead greater modulatory control. Moreover, native promoters could be modified not only in length but also in the arrangement of their response elements, whose number and position may be changed.

Fish expressing GH-encoding transgenes eutopically or even ectopically but at a low level, as recommended by de la Fuente *et al.* (1997), may better reconcile growth enhancement with the preservation of a normal phenotype. In Mediterranean cultured fish, more physiological promoters could be coupled to species-specific GH-encoding DNAs, although this may complicate somehow the assessment of transgene integration. In fact, the cDNA sequence of GH has been determined in both seabream (Funkenstein *et al.*, 1991) and seabass (Doliana *et al.*, 1992). Transgenic fish bearing species-specific constructs with eutopic expression, if produced in the future, would be autotransgenic, according to the definition by Beardmore (1997), and might be even considered for use as broodstock in selective

breeding programs. In this case, transgenetics would blend with quantitative genetics.

Finally a brief remark about fairness in fish transgenesis, an issue that amounts to whether and how to inform consumers about fish that have been genetically engineered. As for other transgenic products, an obvious marketing strategy is to avoid any publicity on the grounds that only transgenic fish that are nutritionally safe can be approved for sale. Since this statement is reasonably true, any label attesting the type of production technology seems indeed redundant and possibly a source of confusion and anxiety on the part of the consumers. Yet, this approach is unfair. It is manipulative, opportunistic and intimately distrusting both the product and the consumer. Perhaps one day, we shall realize that we all belong to a society of consumers and that we deserve respectful information not just merely on transgenics but on any product.

Conclusion

In conclusion, aquatic biotechnology is expected to provide a fundamental contribution to the foreseeable development of fish culture. In this regard, the Mediterranean cultured fish represent a stimulating challenge for two main reasons: their biology is more difficult to approach than that of freshwater fishes and thus requires a more sophisticated technological level; and any achievement in these species is likely to open new avenues for the expansion of mariculture and the appreciation of its enormous potential for mankind. In this endeavour, the scientific community needs not only the ingenuity, dedication, prudence and internal criticism of its members but also an informed perception and support by the general public.

There is a tremendous beauty concealed in marine life with its generous trove of gifts to our existence. Aquatic biotechnology is an attempt to enrich this beauty with a small sign of human intelligence, perhaps to enlarge the significance of our fleeting time, more probably to watch ourselves as a part of a miracle.

Acknowledgements

Reviewed work from the authors' laboratories was supported by grants within the Third and Fourth Triennial Plans on Fisheries and Aquaculture in Marine and Brackish Waters, Ministry of Agricultural Policies, Italy, and the RAISA programme of the National Research Council of Italy.

References

Aleström, P., Kisen, G., Klungland, H. and Anderson, O. (1992). Fish gonadotropin-releasing hormone gene and molecular approaches for control of sexual maturation: Development of a transgenic fish model. *Mol. Mar. Biol. Biotechnol.*, 1: 376-379.

Aliah, R.S., Inada, Y., Yamaoka, K. and Taniguchi, N. (1991). Effects of triploidy on

hematological characteristics and oxygen consumption in ayu. *Nippon Suisan Gakkaishi*, 57: 833-836.

Andersen, B. and Rosenfeld, M.G. (1994). Pit-1 determines cell types during development of the anterior pituitary gland. *J. Biol. Chem.*, 269: 29335-29338.

Anderson, E.D., Mourich, D.V., Fahrenkrug, S.C., LaPatra, S. and Leong, J. (1996). Genetic immunization of rainbow trout (Oncorhynchus mykiss) against infectious hematopoietic necrosis virus. *Mol. Mar. Biol. Biotechnol.*, 5: 114-122.

Arai, K., Ikeno, M. and Suzuki, R. (1995). Production of androgenetic diploid loach *Misgurnus anguillicaudatus* using spermatozoa of natural tetraploids. *Aquaculture*, 137: 131-138.

Argenton, F., Vianello, S., Bernardini, S., Jacquemin, P., Martial, J., Belayew, A., Colombo, L. and Bortolussi, M. (1993). The transcriptional regulation of the growth hormone gene is conserved in vertebrate evolution. *Biochem. Biophys. Res. Commun.*, 192: 1360-1366.

Argenton, F., Bernardini, S., Puttini, S., Colombo, L. and Bortolussi, M. (1996a). A TGACG motif mediates growth-hormone-factor-1/pituitary-transcriptional-activator-1-dependent cAMP reglation of the rainbow trout growth-hormone promoter. *Eur. J. Biochem.*, 238: 591-598.

Argenton, F., De Mas, S., Malocco, C., Dalla Valle, L., Giorgetti, G. and Colombo, L. (1996b). Use of random DNA amplification to generate specific molecular probes for hybridization tests and PCR-based diagnosis of *Yersinia ruckeri*. *Dis. Aquat. Org.*, 24: 121-127.

Barbaro, A., Belvedere, P., Borgoni, N., Bozzato, G., Francescon, A., Libertini, A., Meneghetti, F., Merlin, A. and Colombo, L. (1996). Chromosome set manipulation in the gilthead seabream (*Sparus aurata* L.). In: *Proceedings of the International Workshop on Seabass and Seabream Culture: Problems and Prospects*, Chatain, B., Saroglia, M., Sweetman, J. and Lavens, P. (eds). International Workshop, Verona, Italy, 16/18-10-1996. European Aquaculture Society, Oostende, Belgium, pp. 227-230

Barbaro, A., Francescon, A., Libertini, A., Bozzato, G., Nassi, M., Calderazzo, F. and Colombo, L. (1998). Manipolazione cromosomica nel branzino, *Dicentrarchus labrax* L.: Osservazioni a breve e medio termine sulla progenie ottenuta. *Biologia Marina Mediterranea*, in press.

Baroiller, J.F., Nakayama, I., Foresti, F. and Chourrout, D. (1996). Sex determination studies in two species of teleost fish, *Oreochromis niloticus* and *Leporinus elongatus*. *Zool. Studies.*, 35: 279-285.

Beardmore, J.A. (1997). Transgenics: Autotransgenics and allotransgenics. *Transgenic Res.*, 6: 107-108.

Belvedere, P., Dalla Valle, L., Lucchetti, A., Ramina, A., Vianello, S. and Colombo, L.

(1997). Extraglandular expression of genes encoding steroidogenic cytochromes in rainbow trout (*Oncorhynchus mykiss* Walbaum). *Annals N.Y. Acad. Sc.* (in press).

Blázquez, M., Piferrer, F., Zanuy, S., Carrillo, M. and Donaldson, E.M. (1995). Development of sex control techniques for European sea bass (*Dicentrarchus labrax* L.) aquaculture: Effect of dietary 17α-methyltestosterone prior to sex differentiation. *Aquaculture,* 135: 329-342.

Bongers, A.B.J., Nguenga, D., Eding, E.H. and Richter, C.J. (1995). Androgenesis in the African catfish, *Clarias gariepinus*. *Aquatic Living Resources*, 8: 329-332.

Botsford, L.W., Castilla, J.C. and Peterson, C.H. (1997). The management of fisheries and marine ecosystems. *Science*, 277: 509-514.

Bramick, U., Puckhaber, B., Langholz, H.J. and Horstgen-Schwark, G. (1995). Testing of triploid tilapia (*Oreochromis niloticus*) under tropical pond conditions. *Aquaculture,* 137: 343-353.

Buth, D.G., Gordon, M.S., Plaut, I., Drill, S.L. and Adams, L.G. (1995). Genetic heterogeneity in isogenic homozygous clonal zebrafish. *Proc. Natl. Acad. Sc. USA*, 92: 12367-12369.

Campton, D.E. (1987). Natural hybridization and introgression in fishes. In: *Population Genetics & Fishery Management*. Ryman, N. and Utter, F. (eds). University of Washington Press, pp. 161-192.

Carrillo, M., Zanuy, S., Blázquez, M., Ramos, J., Piferrer, F. and Donaldson, E.M. (1993). Sex control and ploidy manipulation in sea bass. Abstract of the WAS Conference and Trade Show, Torremolinos, Spain, p. 512.

Carrillo, M., Zanuy, S., Prat, F., Cerdá, J., Ramos, J., Mañanós, E. and Bromage, N.R. (1995). Sea bass (*Dicentrarchus labrax*). In: *Broodstock Management and Egg and Larval Quality*. Bromage, N.R. and Roberts, R.J. (eds). Blackwell Science, Oxford, pp. 138-168.

Carter, C.G., McCarthy, I.D., Houlihan, D.F., Johnstone, R., Walsingham, M.V. and Mitchell, A.I. (1994). Food consumption, feeding behaviour, anf growth of triploid and diploid Atlantic salmon, *Salmo salar* L., parr., *Can. J. Zool.*, 72: 609-617.

Chen, T.T., Lu, J.K., Shamblott, M.J., Cheng, C.M., Lin, C.M., Burns, J.C., Reimschuessel, R., Chatakondi, N. and Dunham, R.A. (1995). Transgenic fish: ideal models for basic research and biotechnological applications. *Zool. Stud.*, 34: 215-234.

Cherfas, N., Gomelski, B.I., Emelyanova, O.V. and Recoubratsky, A.V. (1994). Induced diploid gynogenesis and polyploidy in crucian carp, *Carassius auratus gibelio* (Bloch), x common carp, *Cyprinus carpio* L., hybrids. *Aquacul. Fish. Manag.*, 25: 943-954.

Cherfas, N., Gomelsky, B., Ben-Dom, N. and Hulata, G. (1995). Evidence for the heritable nature of spontaneous diploidization in common carp, *Cyprinus carpio* L., eggs. *Aquacul. Res.*, 26: 289-292.

Chevassus, B. (1979). Hybridization in salmonids: Results and perspectives. *Aquaculture,* 17: 113-128.

Chevassus, D. (1987). Genetic manipulation in fish: Review of methods. In: *Proc. World Symp. on Selection, Hybridization and Genetic Engineering in Aquaculture of Fish and Shellfish for Consumption and Stocking*, Bordeaux, France, 27-30 May 1986, Tiews, K. (ed). Berlin, Vol. II, pp. 111-126.

Choubert, G. and Blanc J.-M. (1985). Flesh colour of diploid and triploid rainbow trout (*Salmo gairdneri* Rich.) fed canthaxanthin. *Aquaculture*, 47: 299-304.

Chourrout, D. (1986). Techniques of chromosome manipulation in rainbow trout: A new evaluation with caryology. *Theor. Appl. Genet.*, 72: 627-632.

Chourrout, D. (1987). Genetic manipulations in fish: Review of methods. In: *Proceedings of the World Symposium on Selection, Hybridization, and Genetic Engineering in Aquaculture*, Bordeaux, 207-30 May, 1986, Tiews, K. (ed). Berlin, Vol. II, pp 111-126.

Chourrout, D. and Perrot, E. (1992). No transgenic rainbow trout produced with sperm incubated with linear DNA. *Mol. Marine Biol. Biotechnol.*, 1: 282-285.

Collodi, P., Kamei, Y., Sharps, A., Weber, D. and Barnes, D. (1992). Fish embryo cell cultures for derivation of stem cells and transgenic chimeras. *Mol. Mar. Biol. Biotechnol.*, 1: 257-265.

Colombo, L., Pickering, D.A., Belvedere, P. and Schreck, C.B. (1990). Stress inducing factors and stress reaction in aquaculture. In: *Aquaculture Europe '89 - Business Joins Science*, De Pauw, N. and Billard, R. (eds). European Aquaculture Society, Special Publication No. 12, Bredene, Belgium, pp. 1-29.

Colombo, L., Barbaro, A., Libertini, A., Benedetti, P., Francescon, A. and Lombardo, I. (1995). Artificial fertilization and induction of triploidy and meiogynogenesis in the European sea bass, *Dicentrarchus labrax* L. *J. Appl. Ichthyol.*, 11: 118-125.

Colombo, L., Barbaro, A., Francescon, A., Libertini, A., Benedetti, P., Dalla Valle, L., Pazzaglia, M., Pugi, L., Argenton, F., Bortolussi, M. and Belvedere, P. (1996). Potential gains through genetic improvements: Chromosome set manipulation and hybridization. In: *Proceedings of the International Workshop on Seabass and Seabream Culture: Problems and Prospects*, Chatain, B., Saroglia, M., Sweetman, J. and Lavens, P. (eds). International Workshop, Verona, Italy, 16/18-10-1996, published by European Aquaculture Society, Oostende, Belgium, pp. 343-362.

Curatolo, A., Gunnella, F., Santulli, A., Wilkins, N.P. and D'Amelio, V. (1996). Chromosome set manipulation in the European sea bass (*Dicentrarchus labrax*

L.): Preliminary results. In: *Proceedings of the International Workshop on Seabass and Seabream Culture: Problems and Prospects*, Chatain, B., Saroglia, M., Sweetman, J. and Lavens, P. (eds). International Workshop, Verona, Italy, 16/18-10-1996, published by European Aquaculture Society, Oostende, Belgium, pp. 262-265.

De la Fuente, J., Guillén, I. and Estrada, M.P. (1997). The paradox of growth in fish. Proceedings of the 4th International Marine Biotechnology Conference, Sorrento, Italy, 22-29-09-1997, abstract No. 19.

del Valle, G., Taniguchi, N. and Tsujimura, A. (1994). Reduced variation of physiological traits in ayu clones, *Plecoglossus altivelis*. *Fish. Sc.*, 60: 523-526.

Detimers, J.M., Devries, D.R. and Stein, R.A. (1996). Quantifying responses to hybrid striped bass predation across multiple trophic levels: Implications for reservoir biomanipulation. *Trans. Am. Fish. Soc.*, 125: 491-504.

Devlin, R.H., Yesaki, T.Y., Biagi, C.A., Donaldson, E.M., Swanson, P. and Chan, W.-K. (1994). Extraordinary salmon growth. *Nature,* 371: 209-210.

Diskin, K. (ed). (1993). The National Center for Mariculture. In *Israel Oceanographic & Limnological Research*, Biennial Report 1992-1993, Copying Centre Ltd, Haifa, Israel, pp. 63-73.

Disney, J.E., Johnson, K.R. and Thorgaard, G.H. (1987). Intergeneric gene transfer of six isozyme loci in rainbow trout by sperm chromosome fragmentation and gynogenesis. *J Exp. Zool.*, 244: 151-158.

Disney, J.E., Johnson, K.R., Banks, D.K. and Thorgaard, G.H. (1988). Maintenance of foreign gene expression in adult transgenic rainbow trout and their offspring. *J. Exp. Zool.*, 248: 335-344.

Doliana, R., Bortolussi, M. and Colombo, L. (1992). Cloning and sequencing of European sea bass (*Dicentrarchus labrax* L.) growth hormone cDNA using polymerase chain reaction and degenerate oligonucleotides. DNA sequence. *Journal of DNA Sequencing and Mapping*, 3: 185-189.

Du, S.J., Gong, Z., Fletcher, G.L., Shears, M.A., King, M.J., Idler, D.R. and Hew, C.L. (1992). Growth enhancement in transgenic Atlantic salmon by the use of an "all-fish" chimeric growth hormone gene construct. *Bio/Technology*, 10: 176-181.

Dujakovic, J.J. and Glamuzina, B. (1990). Intergeneric hybridization in Sparidae. 1. *Sparus aurata* female X *Diplodus puntazzo* male and *Sparus aurata* female X *Diplodus vulgaris* male. *Aquaculture,* 86: 369-378.

Dujakovic, J.J. and Glamuzina, B. (1993). Intergeneric hybridization in Sparidae. 2. *Diplodus sargus* female X *Dentex dentex* male. *J. Appl. Aquacul.*, 2: 105-114.

FAO (1994). Review of the State of World Marine Fisheries Management, FAO Tech. Paper 335.

FAO (1995). Fishery Information, Data and Statistics Service. Aquaculture production statistics. FAO Fisheries Circular No. 815, p. 186.

Felip, A., Zanuy, S., Carrillo, M., Ramos, J. and Piferrer, F. (1997). Optimal conditions for triploidy induction in the sea bass (*Dicentrarchus labrax* L.). *Aquaculture* (In press).

Fjalestad, K. T., Gjedrem, T. and Gjerde, B. (1993). Genetic improvement of disease resistance in fish: An overview. *Aquaculture*, 111: 65-74.

Fletcher, G.L., Davies, P.L. and Hew, C.L. (1992). Genetic engineering of freeze-resistant Atlantic salmon. In: *Transgenic Fish*, Hew, C.L. and Fletcher, G.L. (eds). World Scientific, pp. 190-208.

Francescon, A., Libertini, A., Barbaro, A., Bozzato, G. and Lombardo, I. (1994). Prime sperimentazioni di manipolazione cromosomica in orata (*Sparus aurata* L.). *Biol. Mar. Medit.*, 2: 317-318.

Fries, L.T. and Harvey, W.D. (1989). Natural hybridization of white bass and yellow bass in Texas. *Trans. Am. Fish. Soc.*, 118: 87-89.

Funkenstein, B., Chen, T.T., Powers, D.A. and Cavari, B. (1991). Cloning and sequencing of the gilthead seabream (*Sparus aurata*) growth hormone-encoding cDNA. *Gene*, 103: 243-247.

Gallagher, M.L. (1994). The use of soybean meal as a replacement for fish meal in dieats for hybrid striped bass (*Morone saxatilis* x *M. chrysops*). *Aquaculture*, 126: 119-127.

Garrido-Ramos, M., de la Herrán, R., Lozano, R., Cárdenas, S., Ruiz Rejón, C. and Ruiz Rejón, M. (1996). Induction of triploidy in offspring of gilthead seabream (*Sparus aurata*) by means of heat shock. *J. Appl. Ichthyol.*, 12: 53-55.

Gibbs, P.D.L., Peek, A., and Thorgaard, G. (1994). An *in-vivo* screen for the luciferase transgene in zebrafish. *Mol. Mar. Biol. Biotechnol.*, 3: 307-316.

Gong, Z. and Hew, C.L. (1995). Transgenic fish in aquaculture and development biology. *Cur. Top. Dev. Biol.*, 30: 177-214.

Gorshkova, G., Gorshkov, S., Hadani, A., Gordin, H. and Knibb, W. (1995). Chromosome set manipulation in marine fish. *Aquaculture,* 137: 157-158.

Gorshkova, G.V., Gorshkov, S., Gordin, H. and Knibb, W.R. (1996). Sex control and gynogenetic production in European sea bass *Dicentrarchus labrax*. In: *Proceedings of the International Workshop on Seabass and Seabream Culture: Problems and Prospects*, Chatain, B., Saroglia, M., Sweetman, J. and Lavens, P. (eds). International Workshop, Verona, Italy, 16/18-10-1996, published by European Aquaculture Society, Oostende, Belgium, pp. 288-290.

Goudie, C.A., Simco, B.A., Davis, K.B. and Liu, Q.H. (1995). Production of

gynogenetic and polyploid catfish by pressure-induced chromosome set manipulation. *Aquaculture,* 133: 185-198.

Grobet, L., Martin, L.J.R., Poncelet, D., Pirottin, D., Brouwers, B., Riquet, J., Schoeberlein, A., Dunner, S., Ménissier, F., Massabanda, J., Fries, R., Hanset, R. and Georges, M. (1997). A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nat. Genet.,* 17: 71-74.

Gui, J., Liang, S., Zhu, L., Sun, J. and Jiang, Y. (1993). Cytogenetic analysis of developmental differences between embryos of reciprocal cross in distant hybrids of fish. *Zool. Res.*, 14: 171-177.

Hallermann, E.M. and Kapuscinski, A.R. (1992). Ecological implications of using transgenic fishes in aquaculture. *ICES Mar. Sci. Symp.*, 194: 56-66.

Hallerman, E.M. (1994). Towards coordination and funding of long-term genetic improvement programs for striped and hybrid bass *Morone* sp. *J. World Aquacul. Soc.*, 25: 360-365.

Hara, M., Dewa, K. and Yamamoto, E. (1993). DNA fingerprinting with non-radioactive probe in clonal flounder *Paralichthys olivaceous*. *Nippon Suisan Gakkaishi*, 59: 731.

Harache, Y. and Paquotte, P. (1996). The development of marine fish farming in Europe: A parallel with salmon culture. In: *Proceedings of the International Workshop on Seabass and Seabream Culture: Problems and Prospects*, Chatain, B., Saroglia, M., Sweetman, J. and Lavens, P. (eds). International Workshop, Verona, Italy, 16/18-10-1996, published by European Aquaculture Society, Oostende, Belgium, pp. 369-378d.

Henderson-Arzapalo, A., Colura, R.L., Maciorowski, A.F. (1994). A comparison of black drum, red drum and their hybrid in saltwater pond culture. *J. World Aquacul. Soc.*, 25: 289-296.

Hindar, K. (1993). Genetically engineered fish and their possible environmental impact. *NINA Oppdragsmelding,* 215: 1-48.

Hoornbeek, F.K. and Burke, P.M. (1981). Induced chromosome number variation in the winter flounder. *J. Hered.,* 72: 189-192.

Horvath, L. and Orban, L. (1995). Genome and gene manipulation in the common carp. *Aquaculture*, 129: 157-181.

Hubbs, C.L. (1955). Hybridization between fish species in nature. *System. Zool.*, 4: 1-20.

Iyengar, A. and Maclean, N. (1995). Transgene concatemerisation and expression in rainbow trout (*Oncorhynchus mykiss*). *Mol. Mar. Biol. Biotechnol.*, 4: 248-254.

Iyengar, A., Müller, F. and Maclean, N. (1996). regulation and expression of

transgenes in fish. *Transgenic Res.*, 5: 147-166.

Jiang, Y. (1993). Transgenic fish - Gene transfer to increase disease and cold resistance. *Aquaculture*, 111: 31-40.

Johnson, K.R., Wright, J.E. and May, B. (1987). Linkage relationships reflecting ancestral tetraploidy in salmonid fish. *Genetics*, 116: 579-591.

Kapuscinski, A.R. and Hallermann, E.M. (1991). Implications of introduction of transgenic fish into natural ecosystems. *Can. J. Fish. Aquat. Sci.*, 48: 99-107.

Khoo, H.-W., Ang, L.-H., Lim, H.-B. and Wong, K.-Y. (1992). Sperm cells as vectors for introducing foreign DNA into zebrafish. *Aquaculture*, 107: 1-19.

Knibb, W., Gorshkova, G. and Gorshkov, S. (1996). Potentials gains through genetic improvement: selection and transgenesis. In: Seabass and Seabream Culture: Problems and Prospects, Chatain, B., Saroglia, M., Sweetman, J. and Lavens, P. (eds), International Workshop, Verona, Italy, 16/18-10-1996, published by European Aquaculture Society, Oostende, Belgium, pp. 175-188.

Knibb, W. (1997). Risk from genetically engineered and modified marine fish. *Transgenic Res.*, 6: 59-67.

Komen, J., van den Dobbelsteen, P.J., Slierendrecht, W.J. and van Muiswinkel, W.B. (1990). Skin grafting in gynogenetic common carp (*Cyprinus carpio*, L.). The development of histocompatible clones. *Transplantation*, 49: 788-793.

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

Kroll, K.L. and Amaya, E. (1996). Transgenic *Xenopus embryos* from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 122: 3173-3183.

Kurita, J., Oshiro, T., Takashima, F. and Sakaizumi, M. (1995). Cytogenetic studies on diploid and triploid oogenesis in interspecific hybrid fish between *Oryzias latipes* and *O. curvinotus*. *J. Exp. Zool.*, 273: 234-241.

Leary, R.F., Allendorf, F.W., Knudsen, K.L. and Thorgaard, G.H. (1985). Heterozygosity and developmental stability in gynogenetic diploid and triploid rainbow trout. *Heredity*, 54: 219-225.

Leclerc, G.M., Ely, B., Xu, X.L. and Harrell, R.M. (1996). Gynogen production in hybrid striped bass. *J. World Aquacul. Soc.*, 27: 119-125.

Lu, R., Chen, H., Gall, G.A.E. and Chen, H.X. (1993). Advances in fish cell engineering in China. *Aquaculture*, 111: 41-50.

Maclean, N. and Rahman, A. (1994). Transgenic fish. In: *Animals with Novel Genes*,

Maclean N. (ed.). Cambridge University Press, pp. 63-105.

Mair, G.C., Abucay, J.S., Beardmore, J.A. and Skibinski, D.O.F. (1995). Growth performance trials 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.

Malakoff, D. (1997). Extinction on the high seas. *Science*, 277: 486-488.

McAndrew, B.J., Rana, K.J. and Penman, D.J. (1993). Conservation and preservation of genetic variation in aquatic organisms. *Rec. Advan. Aquac.*, 4: 295-336.

McEvoy, T.G., Gannon, F. and Sreenan, J.M. (1992). Gene transfer in fish: Potential and practice. *Anim. Biotechnol.*, 3: 211-243.

McKay, L.R., Ihssen, P.E. and McMillan, I. (1992). Growth and mortality of diploid and triploid tiger trout (*Salmo trutta* x *Salvelinus fontinalis*). *Aquaculture*, 106: 239-251.

McKnight, S.L. (1997). Gatekeepers of organ growth. *Proc. Natl. Acad. Sci. USA*, 94: 12249-12250.

McPherron, A.C., Lawler, A.M. and Lee, S.-J. (1997). Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. *Nature*, 387: 83-88.

Müller, F., Ivics, Z., Erdélyi, F., Papp, T., Váradi, L., Horváth, L., Maclean, N. and Orbán, L. (1992). Introducing foreign genes into fish eggs with electroporated sperm as a carrier. *Mol. Marine Biol. Biotechnol.*, 1: 276-281.

Mullerbelecke, A. and Horstgenschwark, G. (1995). Sex determination in tilapia (*Oreochromis niloticus*) sex ratios in homozygous gynogenetic progeny and their offspring. *Aquaculture*,137: 57-65.

Myers, J.M., Penman, D.J., Basavaraju, Y., Powell, S.F., Baoprasertkul, P., Rana, K.J., Bromage, N. and McAndrew, B.J. (1995). Induction of diploid androgenetic and mitotic gynogenetic Nile tilapia (*Oreochromis niloticus* L.). *Theor. Appl. Genet.*, 90: 205-210.

Mylonas, C.C., Magnus, Y., Gissis, A., Klebanov, Y. and Zohar, Y. (1996). Application of controlled release, GnRHa delivery systems in commercial production of white bass x striped bass hybrids (sunshine bass), using captive broodstocks. *Aquaculture*, 140: 265-280.

Naruse, K., Ijiri, K., Shima, A. and Egam, N. (1985). The production of cloned fish in the medaka (*Oryzias latipes*). *J. Exp. Zool.*, 236: 335-342.

New, M.B. (1997a). Aquaculture and capture fisheries: Balancing the scales. *World Aquaculture,* 28 (In press).

New, M.B. (1997b). The global emergence of tropical aquaculture. *Aquaculture Europe*, 24: 41-49.

Niemann, H. (1996). A survey of sperm mediated DNA-transfer in farm animals. *Reprod. Domestic Anim.*, 31: 211-216.

Noga, E.J., Kerby, J.H., King, W., Aucoin, D.P. and Giesbrecht, F. (1994). Quantitative comparison of the stress response of striped bass (*Morone saxatilis*) and hybrid striped bass (*Morone saxatilis* x *Morone chrysops* and *Morone saxatilis* x *Morone americana*). *Am. J. Vet. Res.*, 55: 405-409.

Ohno, S. (1993). Patterns in genome evolution. *Curr. Opin. Genet. Dev.*, 3: 911-914.

Ojolick, E.J., Cusack, R., Benfey, T.J. and Kerr, S.R. (1995). Survival and growth of all female diploid and triploid rainbow trout (Oncorhinchus mykiss) reared at chronic high temperature. *Aquaculture*, 131: 177-187.

Ozato, K., Wakamatsu, Y. and Inoue, K. (1992). Medaka as a model of transgenic fish. *Mol. Mar. Biol. Biotechnol.*, 1: 346-354.

Palti, Y., Li, J.J. and Thorgaard, G.H. (1997). Improved efficiency of heat and pressure shocks for producing gynogenetic rainbow trout. *Progr. Fish. Cult.*, 59: 1-13.

Pandian, T.J. and Varadaraj, K. (1990). Development of monosex females *Oreochromis mossambicus* broodstock by integrating gynogenetic technique with endocrine sex reversal. *J. Exp. Zool.*, 255: 86-96.

Pandian, T.J. and Sheela, S.G. (1995). Hormonal induction of sex reversal in fish. *Aquaculture*, 138: 1-22.

Powers, D.A., Chen, T.T. and Dunham, R.A. (1992). Transgenic fish. In *Transgenesis. Applications of Gene Transfer*, Murray, J.A.H. (ed). John Wiley and sons, pp. 233-249.

Purdom, C.E. (1993a). Hybridization In: *Genetics and Fish Breeding*, Chapman & Hall, London, pp. 158-177.

Purdom, C.E. (1993b). Chromosome engineering. In: *Genetics and Fish Breeding*, Chapman & Hall, London, pp. 204-222.

Quillet, E. (1994). Survival, growth and reproductive traits of mitotic gynogenetic rainbow trout females. *Aquaculture*, 123: 223-236.

Rahman, M.A., Mak, R., Ayad, H. and Maclean, N. (1997). Expression of an exogenous piscine growth hormone gene results in enhanced growth in transgenic tilapia (*Oreochromis niloticus*). Proceedings of the 4th International Marine Biotechnology Conference, Sorrento, Italy, 22-29-09-1997, abstract n. 25.

Research Group of Cytogenetics, Research Group of Somatic Cell Genetics,

Research Group of Nuclear Transplantation (1981). Nuclear transplantation in teleosts. I. Hybrid fish from the nucleus of carp and the cytoplasm of crucian. *Scientia Sin.*, 23: 517-523.

Roblin, C. and Bruslé, J. (1983). Ontogenèse gonadique et différenciation sexuelle du loup Dicentrarchus labrax en conditions d'élevage. *Reprod. Nutr. Dévelop.*, 23: 115-127.

Scheerer, P.D. and Thorgaard, G.H. (1987). Performance and development stability of triploid tiger trout (brown trout x brook trout). *Transactions of the American Fisheries Society,* 116: 92-97.

Scheerer, P.D., Thorgaard, G.H. and Allendorf, W. (1991). Genetic analysis of androgenetic rainbow trout. *J. Exp. Zool.*, 260: 382-390.

Schmidt, K.F. (1997). "No-take" zones spark fisheries debate. *Science*, 277: 489-491.

Schnieke, A.E., Kind, A.J., Ritchie, W.A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, I., Colman, A. and Campbell, K.H.S. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science*, 278: 2130-2133.

Schultz, R.J. (1980). Role of polyploidy in the evolution of fishes. In: *Polyploidy: Biological Relevance*, Lewis, W.H. (ed). Plenum, New York, pp. 313-340.

Shiraishi, M., Fujii, K., Maruyama, T. and Maeda, H. (1993). Basic research on aquaculture of sturgeon - I: Growth and vitellogenesis of hybrid sturgeon between female *Huso huso* and male *Acipenser ruthenus*, so-called "Bester". *Bull. Nat. Res. Inst. Aquacul.*, 22: 27-35.

Soltis, D.E. and Soltis, P.S. (1995). The dynamic nature of polyploid genomes. *Proc. Natl. Acad. Sci. USA*, 92: 8089-8091.

Stoumboudi, M.T. and Abraham, M. (1996). The spermatogenic process in *Barbus longiceps*, *Capoeta damascina* and their natural sterile hybrid (Teleostei, Cyprinidae). *J. Fish. Biol.*, 49: 458-468.

Streisinger, G., Walker, C., Dower, N., Knauber, D. and Singer, F. (1981). Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature*, 291: 293-296.

Suemori, H., Kadodawa, Y., Goto, K., Araki, I., Kondoh, H. and Nakatsuji, N. (1990). A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous β-galactosidase expression. *Cell Diff. Dev.*, 29: 181-186.

Sugama, K., Taniguchi, N. and Nabeshima, H. (1990a). Frequency of second meiotic division segregation in induced gynogenetic diploid of red sea bream. In: *The Second Asian Fisheries Forum*, Hirano, R. and Hanyu, I. (eds). Asian Fisheries Society, Manila, Philippines, pp. 543-547.

Sugama, K., Taniguchi, N., Seki, S., Nabeshima, H. and Hasegawa, Y. (1990b). Gynogenetic diploid production in the red sea bream using UV-irradiated sperm of black sea bream and heat shock. *Nippon Suisan Gakkaishi,* 56: 1427-1433.

Sugama, K., Taniguchi, N., Seki, S. and Nabeshima, H. (1992). Survival growth and gonadal development of triploid red sea bream, *Pagrus major* (Temminck et Schlegel): Use of allozyme markers for ploidy and family identification. *Aquacul. Fish. Manag.*, 23: 149-159.

Sun, L., Bradford, C.S., Ghosh, C., Collodi, P. and Barnes, D.W. (1995). ES-like cell cultures derived from early zebrafish embryos. *Mol. Mar. Biol. Biotechnol.*, 4: 103-199.

Tacon, A.G.J. (1995). Aquaculture feeds and feeding in the next millenium: major challenges and issues. *FAO Aquaculture Newsletter*, 10: 2-8.

Tacon, A.G.J. (1996). Trends in aquaculture production, with particular reference to low-income food-deficit countries 1984-1993. *FAO Aquaculture Newsletter*, 12: 6-9.

Taniguchi, N., Hatanaka, H. and Seki, S. (1990). Genetic variation in quantitative characters of meiotic- and mitotic-gynogenetic diploid ayu, *Plecoglossus altivelis*. *Aquaculture*, 85: 223-233.

Taniguchi, N., Yamasaki, M., Takagi, M. and Tsujimura, A. (1996). Genetic and environmental variances of body size and morphological traits in communally reared clonal lines from gynogenetic diploid ayu, *Plecoglossus altivelis*. *Aquaculture*, 140: 333-341.

Tave, D. (1993). Growth of triploid and diploid bighead carp, *Hypophthalmichthys nobilis*. *J. Appl. Aquacul.*, 2: 13-25.

Thorgaard, G.H. (1983). Chromosome set manipulation and sex control in fish. In: *Fish Physiology*, Hoar, W.S., Randall, D.J. and Donaldson, E.M. (eds). Vol. 9B, Academic Press, New York, NY, pp. 405-434.

Thorgaard, G.H., Allendorf, F.W. and Knudsen, K.L. (1983) Gene-centromere mapping in rainbow trout: High interference over long map distances. *Genetics*, 103: 771-783.

Thorgaard, G.H. and Allen, S.K. (1987). Chromosome manipulation and markers in fishery management. In: Population Genetics & Fishery Management, Ryman, N. and Utter, F. (eds), University of Washington Press, pp. 319-331.

Thorgaard, G.H., Scheerer, P.D., Hershberger, W.K. and Myers, J.M. (1990). Androgenetic rainbow trout producing using sperm from tetraploid males show improved survival. *Aquaculture*, 85: 215-221.

Thorgaard, G.H., Scheerer, P.D. and Zhang, J. (1992). Integration of chromosome

set manipulation and transgenic technologies for fishes. *Mol. Mar. Biol. Biotech.*, 1: 251-256.

Thorgaard, G.H., Spruell, P., Wheeler, P.A., Scheerer, P.D., Peek, A.S., Valentine, J.J. and Hilton, B. (1995). Incidence of albinos as a monitor for induced triploidy in rainbow trout. *Aquaculture*, 137: 121-130.

Tiews, K. 1987. Selection, Hybridization and Genetic Engineering. In: *Aquaculture*, Heeneman Verlags Gesellschaft mbH (eds). Berlin, vol. 1 and 2.

Trombka, D. and Avtalion, R. (1993). Sex determination in tilapia - A review. *Israeli J. Aquacul.*, 45: 26-37.

Tsujimura, A. and Taniguchi, N. (1996). Reduction of phenotypic variation in reproductive traits of clonal strains of ayu *Plecoglossus altivelis*. *Nippon Suisan Gakkaishi*, 62: 393-398.

Varadaraj, K. (1993). Production of viable haploid *Oreochromis mossambicus* gynogens using UV-irradiated sperm. *J. Exp. Zool.*, 267: 460-467.

Volckaert, F.A.M., Galbusera, P.H.A., Hellemans, B.A.S., van den Haute, C., Vanstaen, D. and Ollevier, F. (1994a). Gynogenesis in the African catfish (*Clarias gariepinus*). I. Induction of meiogynogenesis with thermal and pressure shocks. *Aquaculture,* 128: 221-133.

Volckaert, F.A., Hellemans, B.A., Galbusera, P., Sekkali, B., Belayew, A. and Ollevier, F. (1994b). Replication, expression, and fate of foreign DNA during embryonic and larval development of the African catfish (*Clarias gariepinus*). *Mol. Mar. Biol. Biotechnol.*, 3: 57-69.

von Lukowicz, M., Forster, M., Hilge, V., Klupp, R., Rosch, R., Schultze, D., Stein, H., Ungemach, H. and von Lukowicz, M. (1990). Genetic manipulation of fresh water fish. Stage of development, importance and ecological risks. *Bayerisches Landwirtschaftliches Jahrbuch,* 67: 771-781.

Wang, R., Zhang, P., Gong, Z. and Hew, C.L. (1995). Expression of the antifreeze protein gene in transgenci goldfish (*Carassius auratus*) and its implication in cold adaptation. *Mol. Mar. Biol. Biotechnol.*, 4: 20-26.

Ward, R., Blandon, I.R. and Bumguardner, B.W. (1995). Hybridization among members of the genus *Morone* (Pisces, Percichthyidae) in Galvestone Bay, Texas. *Texas J. Sci.*, 47: 155-158.

Wiegertjes, G.F., Groeneveld, A. and Vanmuiswinkel, W.B. (1995). Genetic variation in susceptibility to *Trypanoplasma borrelli* infection in common carp (*Cyprinus carpio*). *Vet. Immunol. Immunop.*, 47: 153-161.

Wiegertjes, G.F., Stet, R.J.M., Parmentier, H.K. and Vanmuiswinkel, W.B. (1996). Immunogenetics of disease resistance in fish: A comparative approach. *Dev. Comp. Immunol.*, 20: 365-381.

Williams, D.W., Müller, F., Lavender, F.L., Orbán, L. and Maclean, N. (1996). High transgene activity in the yolk syncytial layer affects quantitative transient expression assays in zebrafish (*Danio rerio*) embryos. Transgenic Res., 5: 433-442.

Wohlfarth, G.W. (1994). The unexploited potential of tilapia hybrids in aquaculture. *Aquacul. Fish. Manag.*, 25: 781-788.

Wolters, W.R. and Demay, R. (1996). Production characteristics of striped bass x white bass and striped bass x yellow bass hybrids. *J. World Aquacul. Soc.*, 27: 202-207.

Woods, L.C., Ely, B., Leclerc, G. and Harrell, R.M. (1995a). Evidence for genetic purity of captive and domestic striped bass broodstocks. *Aquaculture*, 137: 41-44.

Woods, L.C., Hohler, C.C., Sheehan, R.J. and Sullivan, C.V. (1995b). Volitional tank spawning of female striped bass with male white bass produces hybrid offspring. *Trans. Am. Fish. Soc.*, 124: 628-632.

Yamamoto, A. and Iida, T. (1994). Hematological characteristics of triploid rainbow trout. *Gyobyo-Kenkyu*, 29: 239-243.

Yamamoto, A. and Iida, T. (1995). Bactericidal activity of serum of all female triploid rainbow trout. *Fish Pathol.*, 30: 123-124.

Yamazaki, F. (1983). Sex control and manipulation in fish. *Aquaculture*, 33: 329-354.

Yiengar, A., Müller, F. and Maclean, N. (1996). Regulation and expression of transgenes in fish - A review. *Transgenic Res.*, 5: 147-166.

Young, W.P., Wheeler, P.A. and Thorgaard, G.H. (1995). Asymmetry and variability characters and spotting in isogenic lines of rainbow trout. *Aquaculture*, 137: 67-76.

Young, W.P., Wheeler, P.A., Fields, R.D. and Thorgaard, G.H. (1996). DNA fingerprinting confirms isogenicity of androgenetically derived rainbow trout lines. *J. Hered.*, 87: 77-81.

Zanuy, S., Carrillo, M., Blázquez, M., Ramos, J., Piferrer, F. and Donaldson, E.M. (1994). Production of monosex and sterile sea bass by hormonal and genetic approaches. European Aquaculture Society, Special Publication No. 21: 1-9.

Zhang, Q.Q. and Arai, K. (1996). Flow cytometry for DNA contents of somatic cells and spermatozoa in the progeny of natural tetraploid loach. *Fish. Sci.*, 62: 870-877.

Zhu, Z., Li, G. and Chen, S. (1985). Novel gene transfer into the fertilized eggs of goldfish (Carassius auratus L. 1758). *Z. Angew. Ichthyol.*, 1: 31-34.