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Biotechnology applied to salmoniculture

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ABSTRACT- This work presents a conceptual analysis of the main biotechnologies used in Chilean salmoniculture, which is based on the production of Atlantic salmon (*Salmo salar*, 356.407 t), silver salmon or coho (*Oncorhynchus kisutch*, 116.481 t), (*O. tshawytscha*, 2.062 t) and rainbow trout (*O. mykiss*, 189.178 t). These activities are focused on the photoperiod artificial manipulation to obtain out-of-season spawning, in the use of hormonal therapies which allow synchronizing the final oocyte maturation (FOM) and sexual maturity acceleration or the increase in milt volume produced by males. Such actions are carried out using GnRHa in doses close to 10 µg/kg of fish. Once sexual maturity is reached, *in vitro* manipulation of gamets must often be done due to either the prolonged storage (particularly milt) they have to undergo in order to transport them where fertilization takes place, or awaiting for the ichtiopathological results, usually taken to broodstock. The production of "all female" populations is also common. Frequently, these populations in rainbow trout are triploided (through shock temperature close to 28º C or pressure close to 8.000 psi) to obtain sterile species which improve productive perfomance of cultured populations without sexual maturity signs. Besides, the perspectives of industrial use of transgenic organisms in the culture of salmonids are analyzed.

Key Words: biotechnology, induced spawning, photoperiod, reproduction, sex manipulation, thermoperiod

Biotecnologia aplicada à salmonicultura

RESUMO - Este trabalho apresenta uma análise conceitual das principais biotecnologias utilizadas na salmonicultura chilena, que é baseada na produção de salmão-do-Atlântico (Salmo*salar*, 356,407 t), o prateado ou salmão coho (*Oncorhynchus kisutch*, 116,481 t), (*O. tshawytscha*, 2,062 t) e truta arco-íris (*O. mykiss*, 189,178 t). Estas atividades centram-se na manipulação do fotoperíodo artificial para obter desova fora de época, no uso de terapias hormonais que permitem sincronizar a maturação ovocitária final (FOM) e aceleração da maturidade sexual ou aumento do volume de sêmen produzido pelos machos. Essas ações são realizadas com GnRHa em doses próximas de 10ìg/kg de peixe. Após a maturidade sexual, a manipulação de gametas "in vitro" deve ser feita, quer seja devido ao armazenamento prolongado (particularmente "milt"), pois têm que ser transportado ao local em que ocorrerá a fecundação, ou para aguardar os resultados ictiopatológicos, geralmente tomada dos reprodutores. A produção de população "todas fêmeas" também é comum. Frequentemente, estas populações em truta arco-íris são triplóides (por meio de choque temperatura próxima a 28 ºC ou pressão próxima de 8.000 psi) para obter espécies estéreis que melhoram desempenho produtivo das populações cultivadas sem sinais de maturidade sexual. Além disso, as perspectivas de utilização industrial de organismos transgênicos na criação de salmonídeos são analisadas.

Palavras-chave: biotecnologia, desova induzida, fotoperíodo, manipulação de sexo, reprodução, termoperiodo

Introduction

A simple definition of biotechnologyindicates that this includes any technique which uses living organisms or some parts of them to produce or modify products, to improve plants or animals and to develop microorganisms for specific purposes. Biotechnology applied to any area of vegetal or animal production, whether aquatic or terrestrial, has the capacity to change the industrial community of the 21st century due to:

- Its potential to produce practically unlimited quantities of substances like never before;

- The possibility to generate products which are normally produced in reduced quantities;

- The availability of products with lower production costs than the ones produced by conventional methods;

- The option of having more secure products than the ones available until now; and

- The possibility of providing products generated from more plentiful and cheaper raw material than the ones used before.

Many areas of biological sciences are playing a crucial role in the development of sustainable aquaculture. In this conference the term biotechology will be used in its broad

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sense, including all of those biological technologies which are essential for the succesful development of salmoniculture, including areas like broodstock managment, oocytes and sperm induction to final maturation, '*in vitro*' manipulation of gametes, chromosome set manipulation of a specie, sex control of cultured species, larvae incubation and culture, development and metamorphosis, nutrition, growth and conversion factors improvement, cultured animals health, genetic aspects such as 'stocks' identification, gene banks and transgenesis. Many of these biotechnologies are focused on early life cycle stages of the species of commercial interest, from gamete manipulation like cryoperservation, transgenesis, chromosome manipulation, clonning and the control of phenotypic expression of sex.

Due to the giving time for the analysis of each of these biotechnologies, in this review will only be analyzed those which show a higher degree of development in Chilean salmoniculture or the ones which show more perspectives for their future application.

Sexual maturity control

Spawning season modification

In fish, like in vertebratres, reproduction is regulated by the brain through gonadotropin releasing hormone (GnRH) which is produced specifically in the hypothalamus (Harvey & Hoar, 1980; Zohar, 1988; Montero & Dufour, 1996; Donaldson, 1997; Peter & Yu, 1997). This decapeptide stimulates gonadotropin production (GtH) through the hypophysis or pituitary. In some fish, dopamine is a negative inhibitor of production and release of GtH from the hypophysis (Zohar, 1988; Redding & Patiño, 1993; Peter & Yu, 1997). Reproduction control done by this gland is through two gonadotropin hormones: follicle-stimulating hormone (FSH or GtH I) which regulates vitelogenesis in female and spermatogenesis in males, and the luteinizing hormone (LH or GtH II), in charge of controlling final oocyte maturation in females (Peter & Yu, 1997).

In salmonids, sexual maturation is controlled in a synergic way between the photoperiod and temperature, which are the environmental factors that determine hormonal cycles that control reproduction. Vitelogenesis is facilitated for photoperiods of long days and "high" temperatures (close to 15ºC). Final oocyte maturation and spawning require photoperiods of short days and temperatures below 10ºC, so if these elementary requirements in production are kept, particularly low temperatures, photoperiod can be easily manipulated at a prolonged low cost or by shortening the daily hours of light; which allows to carry out-of-season spawning, or up to two spwanings a year, particularly in rainbow trout, even though, experiences with Atlantic salmon are becoming more common.

Sexual maturity induction

Many species kept in artificial culture systems undergo important reproductive disfunctions. Commonly, female start gonadal development normally, but they do not reach the final oocyte maturation (FOM), ovulation or spawning. Even though males are more resistant to culture environmental conditions and to the stress produced by these, they g enerate a lower milt volume, or a bad quality one.

Manipulations of some environmental factors such as photoperiod, temperature, salinity, tank volume, sustrate vegetation, etc., can often improve gamete quality. However, in some species hormonal treatments are the unique alternative to achieve adequate reproductive processes in cultured fish.

In the last few years, a variety of hormones and technologies applied to this usage have been successfully used. These methods started in 1930 with the injection of pituitary extracts (with high GtH levels) to induce spawning. Nowadays, many synthetic components of high power of gonadotropin-releasing hormone (GnRHa) are used – which applied from simple injections in saline solution to pellets (10 µg/kg of fish) have solved numerous reproductive problems in many species.

In vitro gamete management

In vitro gamete preservation is assumming an important role in egg production and in genetic management of broodstock in salmoniculture. Storage techniques for a prolonged period of time have not given a feasible solution to egg storage, which is not the case of sperm, with many storage forms which can go from cryopreservation to low temperature storage (Bromage & Roberts, 1995).

The need to keep or preserve sperm from different fish species outside the testicle environment arises from:

- Asynchrony in male and female maturation (McNiven et al., 1993; Bromage & Roberts 1995);

- The higher demand of optimizing the management and efficiency of a broodstock farm (Stoss & Holtz 1983; Christensen & Tiersh 1996);

- The need to carry out genetic studies and genetic heritage preservation studies of endangered species (Stoss

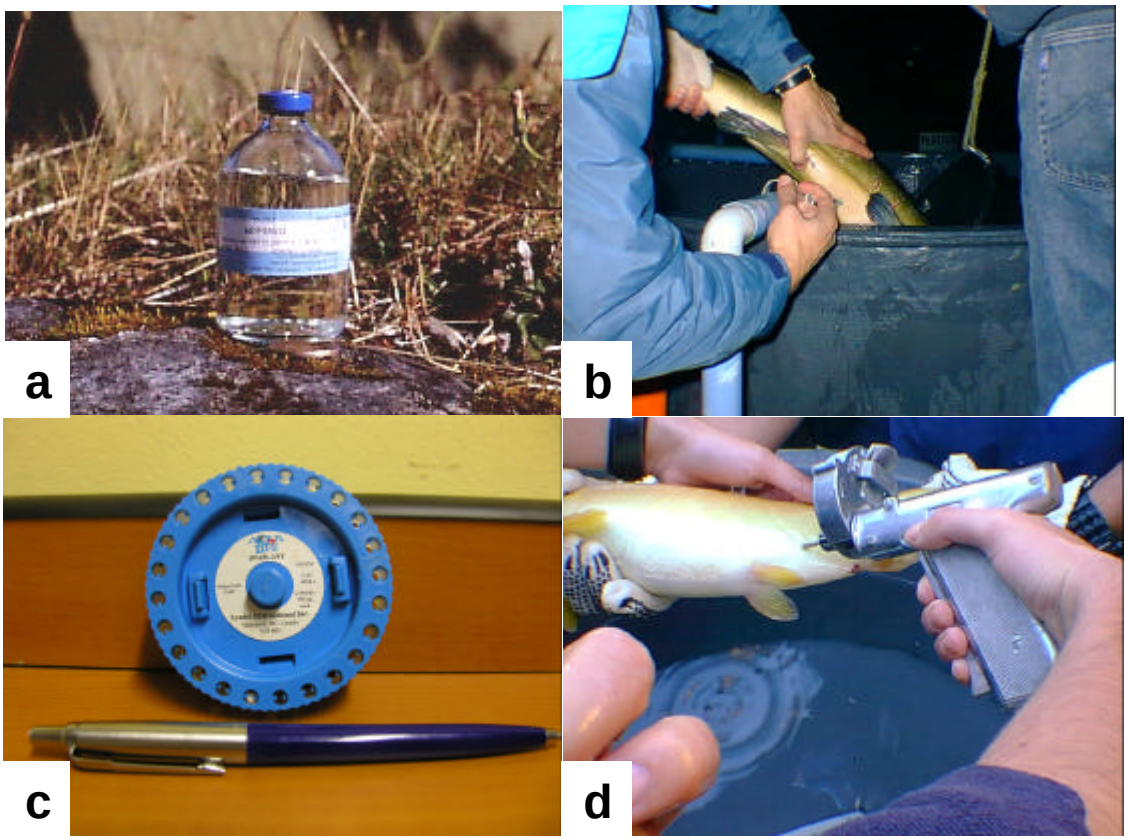

Figure 1 - a: Commercial product (Ovaprim ®) to sexual maturation induction based on GnRH plus a dopamine inhibitor. b: Application of a hormonal dose in Atlantic salmon (*Salmo salar*) through intraperitoneal injection (IP). C: Hormonal implants (Ovaplant ®) used in salmon industry for sexual maturity induction. D: Application of hormonal implants in salmon coho (*Onchorhynchus kisutch*) through IP.

& Refstie, 1983; Korokura et al., 1986, cited by Christensen & Tiersh, 1996);

- The duty of storing sperm, while the icthiopathological diagnosis required to broodstocks is ready, in order to avoid vertical transmission of diseases.

- The need to transfer gametes when the broodstocks are distant from the fish farms where fertilization and incubation take place.

Immobile media

These are isotonic saline solutions which imitate the medium in which sperm is inside the testicle, they are also called “extender, immobile medium or dilutor”. They are regularly used in artificial gamete management in mammals (Sánchez & Rubilar 2001).

Diluents for fish are mainly based on the chemical composition of the seminal plasma of each specie since spermatozoa needs to be kept immobile for a short period of time. Some other components can also be added, like some kind of buffer and glucose, among others (Erdahl & Graham 1987). The chemical composition of these components is very diversed, including the ones recommended for the same specie. Considering this issue, Table 1 shows some examples of some diluents used in salmonid species.

Some researchers have studied the use of a diluent which imitates the content of seminal plasma in fish of commercial interest, mainly trouts and salmons. Truscott & Idler (1969) in Erdahl & Graham (1987), report spermatozoa motility in Atlantic salmon after six days of storage with a diluent which contained chiefly sodium chloride, potasium and calcium, fructose, water and ph 7,3. Erdahl & Graham (1987), stored trout semen for 24 hours using a diluent whose main components were sodium chloride, postasium, magnesium and glucose, with good fertilization results. In a review done by Linhart et al. (1995), the storage of *Polyodon spathula* sperm for a short period is reported using a saline solution of sodium chloride at 0,9%. Conte et

Table 1 - Immobile media used for sperm storage in salmonids

	Component (mM)						
	1	2	3	4	5(g/L)	6	7(g/2L)
NaCl	80	80	110	103	5,16	110	11,7
KCl	40	40	28,3	40	1,64	28,3	5,1
$CaCl_2$	0,1	0,1	-	1,0	0,143	1,8	0,2
NaHCO3	-	-	-	-	1,00	-	-
NaH2PO4	-	-	-	-	0,41	-	-
Na2HPO4	-	-	-	-	-	-	0,5
Tris	30	30	0,02	20	-	0,02	-
$MgSO_4$	-	-	1,1	-	0,223	1,1	-
$MgSO_2$	-	-	-	0,8	-	-	-
MgCl2	-	-	-	-	-	-	0,4
CaCl	-	-	1,8	-	-	-	-
Fructose	-	-	-	-	1,00	-	-
Glucose	-	-	-	-	-	-	20
Citric acid	-	-	-	-	-	-	0,2
Bicine (5,3 g/100 mL)	-	-	-	-	-	-	20 mL
KOH (1,27 g/100 mL)	-	-	-	-	-	-	20 mL
pH	9,2	6,5-8,5	9,0	7,8	7,3	9	7,8
Osmolarity (mOsm)	-	-	-	-	226	-	310

1. Cosson et al., 1999; 2. Woolsey et al., 2006; 3. Billard, 1984; 4. Lahnsteiner et al., 2004; 5. Truscot & Idler, 1969; 6. Billard & Jalabert, 1974; 7. Erdahl & Gram, 1987.

al. (1998) in Linhart et al.(1995), stored sperm from white sturgeon (*Acipenser transmuntanus)* for 14 days at 4ºC.

Bromage & Roberts (1995), report that suministrating oxygen, or the addition of antibiotics together with low storage temperatures, prolong the feasible period of spermatozoa. Similar results were drawn by Valdebenito (2004) for puye sperm storage during three days without significantly losing the fertilizing capacity.

Studies with rainbow trout sperm demonstrated the advisability of combining penicilin and streptomycin to store semen during prolonged periods of time (Stoss & Holtz 1983). Antibiotics are commonly used to protect mammal sperm during storage, but have not been widely used in salmonids.

McNiven et al. (1993), diluted rainbow trout milt in non-aqueous mediums with flurocarbons storing milt even for 37 days with acceptable motility levels. While semen is in a immobile medium, temperature and light must be protected. Regularly, storage takes places in darkness and at a temperature from 0-4ºC (Billard 1984; Stoss & Refstie, 1983; Gordon et al., 1987; Vladic & Jarve, 1997; Lahnsteiner et al., 2004).

Frequently, gaseous oxygen is injected to prolong even more spermatozoa survival and feasability. Gordon et al. (1987), recommends a relation 1:20 between the volume of stored sperm and the gas that covers it. Besides, he indicates that the height of sperm column should not exceed 6 mm. This allows storing semen for several days with good feasibility. Usually, milt is diluted in a relation 1:2 regarding the sperm diluter. During storage, the pH medium is an important factor that determines the movement capacity of spermatozoa after activation. Woolsely et al. (2006), determines that sperm of rainbow trout stored for two hours at pH 8.5 presents a higher percentage of motile sperm than the sperm stored at pH 7.1.

The use of sperm diluents solves somehow the problem of sperm maintenance out of the testicle, allowing to increase the period in which spermatozoa keeps its fertilizing capacity, which contributes enormously for egg broodstocks. This is clear in salmonid fish farmings where it is necessary to preserve the gametes for several days awaiting the 'screening' results taken to each of the males, or to transfer the gametes for many hours when the broodstocks are located far away from the "hactcheries".

Activation media

There are many studies where fish sperm has undergone different extra testicular environments to determine the factors that control its activation and inhibition, feasibility and behaviour. Some solutions used in these researches could be classified as "activation media" since they try to prolong the time and intensity of spermatozoa motility, and therefore its fertilizaing capacity.

In salmonids, the ovarian fluid or celomic liquid which goes with the egg during spawning is a very effective natural activator for spermatozoa (Billard, 1984; Woolsey et al., 2006), so that many spermatic activators used in these species imitate its composition.

Poon & Johnson (1970) cited by Erdahl & Graham (1987), studied the activation of teleost sperm to increase

the number of eggs which can be fertilized using fresh water with unsatisfactory results. Valdebenito (2007) assessed the effect of an activating solution which includes caffeine on sperm motility of rainbow trout, finding out that motility levels increased significantly respect to the control (fresh water) when using a concentration of 3,5 mM of caffeine. Saline solutions have also been largely studied as a possible activation medium and showed more favorable results than in fresh water, but with more limited motility periods (Erdahl & Graham 1987). Goodal et al. (1989), examined some of the factors which regulate activation and sperm motility duration from *Sillago ciliata* directed to spermatozoa fertilizing capacity using solutions which contained sodium chloride, potasium and glucose at different osmolarities, finding out that when using activation mediums which contain sodium chloride and potasium at osmolarities of 600 mOsm, sperm motility increases.

Otha et al. (1997) used saline solutions based on ionic components of seminal plasma from *Anguilla japonica*, modifying the concentrations of some components like potasium, bicarbonate and pH to determine the factors that promote motility on this specie. They found out when increasing the solution concentrations of potasium, bicarbonate and pH from 7,8 to 8,7 the percentage of motile spermatozoa increased.

In production, salmonid fish farms in Chile, regularly use saline solutions or activation media in a proportion close to 20-30% of egg volume in order to carry out fertilization. In this way, sperm motility duration, the intensity of flagelar movement and finally, the fertilizing capacity of sperm are prolonged. Table 2 shows some examples of activation media used in salmonid species.

Sex control

In many fish species like trouts, tilapias, sea bass, and sea bream and in some cases, crustacean, animals from one sex have better productive characteristics than those of the other sex. These features can include more rapid growth, late maturation, or both. For example, an important percentage of males in salmonids maturate before getting into salty water and in average they do that a year before females. Secondary changes caused by maturation (inferior jaw development in the shape of a hook, kind of a hump formation in the dorsal region, lost of orange meat color, growth stoppage, reduction of osmoregulation capacity in sea water and mortality increase), reduce the market value and force the broodstock to harvest before they have reached their total potential growth. On the other hand, females do not experience these problemas, or if the case, with a lower intensity.

The techniques used to modify phenotypic expression of sex in salmonids, are widely used in fish farmings through indirect methods to produce “all female” populations.

In Chile, the production of these populaitons are used widely through the generation of sex-reversed “functional-males” or “neo-males” whose phenotypic expression of sex is altered by providing masculine hormones (regularly 17α methyltestosterone) to the specimens in stages previous to hatch (Pacific salmon) or during the first feeding (troutsand Atlantic salmon). These specimens are characterized for being genetically females (XX), but phenotypically males. Once sexual maturity is reached (after at least two years of culture), the spermatozoa produced are all carriers of chromosome X, for that reason, the sperm obtained from “functional-males” or “neo-males” is called ‘female milt’. When it is used in a fertilization \in protected wild areas, with the aim of avoiding the introduction of exotic species.

In salmonids, triploids are induced avoiding the release of the second polar corpuscle through chemical, physical and electric treatments. Widely, shock temperatures are used (at 28ºC aprox.) (Figure 2a) or pressure (at 10.000 psi) (Figure 2b) applied to eggs after aproximately 30min from fertilization; getting percentages of triploid that regularly exceed 90% of efficiency. In adult salmonids, triploid males are aneuploid and present the same signs of sexual maturity than a normal male (skin darkens, jaw deformation, meat loss of cartenoids and its inmune system gets depressed). However, females do not develop gonades because of their hypothalamus-hypophysis gonade axis is inactive. This makes possible that females keep prepuberty aspects during all their lives, maintaining silver skin, absence of gonadal development, high levels of muscular pigmentation and as they do not spend energy in gonadal development, their growth is aproximately 20% higher than a normal female.

Some of the disadvantages presented in triploid groups are their lower resistence to management conditions and to

Table 2 - Activation media used for sperm motility activation in salmonids

Component (mM)	1	2	3
NaCl	125	125	155
$CaCl_2$	0,1	0,1	-
Tris	30	30	0,02
MgSO4	-	-	1,3
CaCl	-	-	3,4
pH	9,2	8,5	9,0
Others	-	1,0	-

1. Cosson et al., 1999; 2. Woolsey et al., 2006; 3. Billard, 1984.

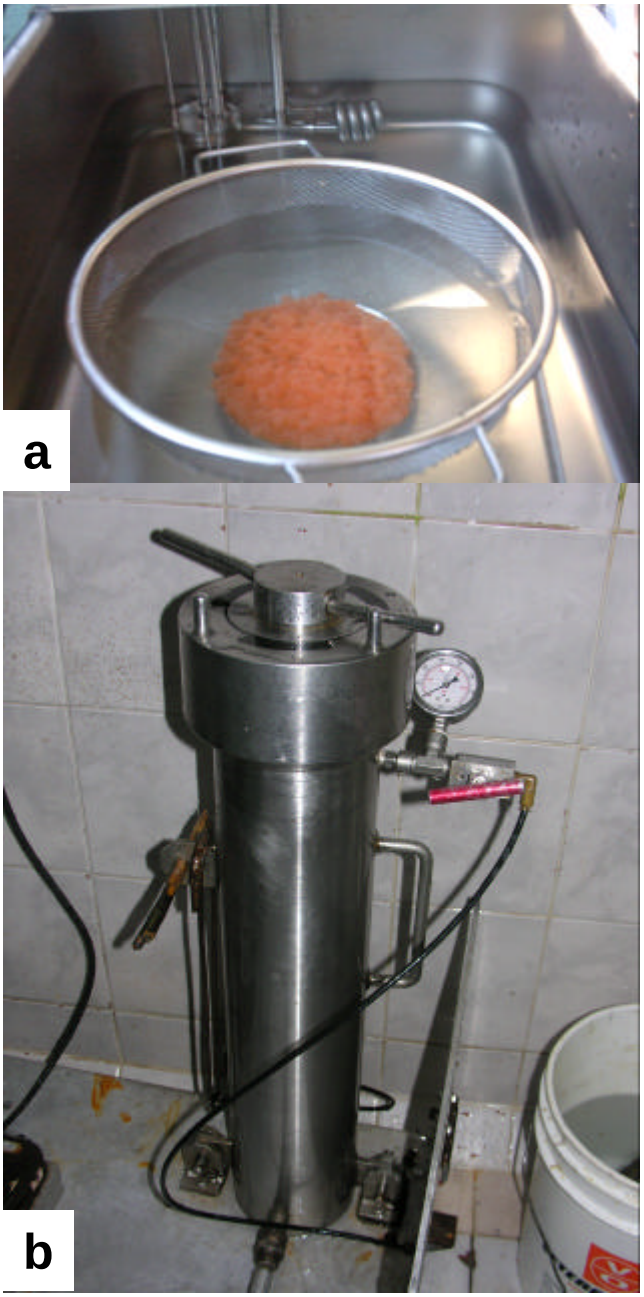

Figure 2 - a) thermic shocks application to recently fertilized eggs from rainbow trout (*Oncorhynchus mykiss*) b) pressure system for triploidy induction.

diseases, as well as higher percentages of deformed specimens.

Due to the productive advantage showed just by triploid females, regularly this technique is applied to functional-males "only female" obtained with some of the methods mentioned previously. This allows having 100% sterile populations, without sexual maturity signs and with harvest alternatives at any time of the year.

Genetics

New technologies of DNA manipulation and specially Polymerase Chain Reaction (PCR), are the base techniques used in the areas of genetics, reproduction, pathogen agents identification and of 'stocks' and disease diagnostic of fish. These techniques allow to quantify the efficiency of genetic selection programs; identify specific genoma sequences of cultured strains which permit to recognize individuals when facing eventual runaways to the environment; determine the sex of specimens even when there are not existent signs of maturity and/or identify the presence of pathogens in asymptomatic individuals.

Transgenic fish

A transgenic organism carries in its genoma copies of fragments of foreign DNA which have been introduced through molecular techniques, so it expresses a phenotypic characteristic that is not present in its specie, or, to intensifies it. Among the many aims in the production of transgenic organisms are the resistance to diseases, tolerance to low temperatures, favourable phenotypic transfer which improve productive indicators such as growth, dietary conversion, production of valuable nutrients for the consumer, like the presence of fatty acids, or the luminiscence emission in aquarium fish.

This biotechnology in salmonids is still in its experimental form, or at small scale, since its application at commercial level is not considered advisable in the short term. However, there are some experiences with transgenic populations in Atlantic salmon where growth in experimental groups has been 11 times higher than the normal groups.

The use of transgenic organisms sets out four very important ethic dilemmas:

1) The necessity that these techniques are used to solve the most urgent problems related to sanitary and diet aspects.

2) The need of distributing these biotechnologies to all countries equally.

3) The necessity of giving the same right of participation to both the benefits and risks of trangenic products to the populations in less favoured regions.

4) The need to cushion the socioeconomic and unfavorable cultural effects the introduction of these organisms can produce in different parts of the populations.

The development in the last ten years of the new powerful biotechnologies, specially the ones related to genetic manipulation of species, has increased extraordinarily the human capacity to manipulate genetic information of cultured organisms, has widened the

acquisition of knowledge and has opened perspectives for application not imaginable before. Nevertheless, fish culture has insufficient elementary research of the species of interest, and therefore that has direct repercussions in the possibilities of application. Some time has to go by until aquaculture activities can equal agriculture or livestock, so the new biotechnologies can be widely applied in this industry, but the future is necessarily optimistic and the possibilities are almost unlimited.

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