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Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment

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Abstract:

Polyploids can be defined as organisms with one or more additional chromosome sets with respect to the number most frequently found in nature for a given species. Triploids, organisms with three sets of homologous chromosomes, are found spontaneously in both wild and cultured populations and can be easily induced in many commercially relevant species of fish and shellfish. The major consequence of triploidy is gonadal sterility, which is of advantage in the aquaculture of molluscs since it can result in superior growth. In fish, the induction of triploidy is mainly used to avoid problems associated with sexual maturation such as lower growth rates, increased incidence of diseases and deterioration of the organoleptic properties. Triploidy can also be used to increase the viability of some hybrids, and is regarded as a potential method for the genetic containment of farmed shellfish and fish. This review focuses on some current issues related to the application of induced polyploidy in aquaculture, namely: 1) the artificial induction of polyploidy and the effectiveness of current triploidisation techniques, including the applicability of tetraploidy to generate auto- and allotriploids; 2) the performance capacity of triploids with respect to diploids; 3) the degree and permanence of gonadal sterility in triploids; and 4) the prospects for the potential future generalised use of triploids to avoid the genetic impact of escapees of farmed fish and shellfish on wild populations. Finally, directions for future research on triploids and their implementation are discussed.

Keywords: Polyploidy; Triploidy; Tetraploidy; Aquaculture; Fish farming; Shellfish; Fish; Sterility; Hybridisation; Reproductive containment; Genetic containment; Transgenic containment; GMO

64 **Contents**

1. Introduction

Sexual maturation usually results in decreased body growth rates since fish divert their energy towards gonadal development. In modern finfish aquaculture, high growth rates cause captive animals to reach puberty earlier than their wild conspecifics (Thorpe, 2004), and occurs during the grow out phase, where high costs of production in food and labour are incurred, and before the fish reach marketable size (Rizzo and Spagnolo, 1996). Moreover, sexual maturation is often associated with higher incidence of diseases, as in the turbot, *Scophthalmus maximus*, or changes in the organoleptic properties of the edible parts, as in many salmonids. These problems can be avoided by producing sterile animals. Sterility may be achieved by the induction of polyploidy, particularly triploidy. Polyploidy is also applied to shellfish species, especially molluscs such as the Pacific oyster, *Crassostrea gigas*, to increase growth rate and/or to improve organoleptic quality (Nell, 2002). Sterility produced by triploidy also has a significant potential applications in the genetic containment of aquaculture species.

Previous reviews on different aspects of triploidy induction and their applications in aquaculture species include those of Purdom (1983), Thorgaard (1983; 1986), Chourrout (1988), Dunham (1990), Ihssen et al. (1990), Beaumont and Fairbrother (1991), Thorgaard et al. (1992), Pandian and Koteesvaran (1998), Benfey (1999), Arai (2001), Felip et al. (2001a), Hulata (2001), Tiwary et al. (2004) and Maxime (2008).

The present review deals first with the production and biology of polyploids in fish and shellfish. We then consider the use of polyploids in aquaculture to improve performance, and explore their value in minimising the potential genetic impacts of farmed molluscs and fish on wild populations.

2. Occurrence of natural animal polyploidy

2.1. Evolutionary incidence of polyploidy in natural populations

Most Metazoa are diploid, possessing a duplicated set of homologous chromosomes in somatic cells, a condition evolved together with gametogenesis via meiotic cell division. Polyploids can be defined as organisms with one or more additional chromosome sets with respect to the number most frequently found in nature for a given species. Polyploidy has been involved in the speciation of both animals and plants (Mable, 2004; Hegarty and Hiscock, 2007), and seems to have arisen independently several times during the evolution of fishes, with higher incidence in the more primitive groups (Legatt and Iwama, 2003). Genetic and epigenetic interactions between redundant genes in polyploid fish (Comai, 2005) have probably influenced their evolutionary fate, leading to their current impressive biological diversity (Le Comber and Smith, 2004). Spontaneous polyploids have been observed in several phylogenetically distant orders, including both wild and farmed fish species (Schulz, 1967; Thorgaard and Gall, 1979).

In the vertebrates, polyploid species are not exclusive to fish, since they have been reported in different groups, from amphibians (Stöck et al., 2002) to occasionally even in mammals (Gallardo et al., 1999). Polyploids can originate either from alterations of meiotic or mitotic processes in specimens within a species (autopolyploidy) or by reproductive contact among species (allopolyploidy).

2.2. Mechanisms of natural autopolyploidy

Spontaneous autopolyploidy arises by multiplication of chromosome sets within a species and occurs by several mechanisms, including: a) derangements of gametogenesis caused by cytogenetic alterations of meiosis, such as pre-meiotic endoduplication of the chromosome set, suppression of the first and/or the second meiotic division (Cherfas et al., 1995a) or nondisjunction of mitotic chromosomes during embryo cleavage; b) suppression of the second meiotic division due to cytoskeletal alterations in post-ovulatory, aged oocytes (Svärdson, 1945; Yamazaki et al., 1989; Cherfas et al., 1991, 1995a; Díaz et al., 1993; Ueda, 1996; Varkonyi et al., 1998; Aegerter and Jalabert, 2004; Aegerter et al., 2004; 2005; Ezaz et al., 2004; Flajšhans et al., 2007) or c) disruption of the process of gamete fertilisation by, for example, polyspermy (Grunina et al., 1995; Recoubratsky et al., 1996, Kirankumar and Pandian, 2004; Grunina et al., 2006).

Some of these disruptions of the normal meiotic process that lead to polyploidisation have become evolutionarily fixed and made possible the development of polyploid *taxa* of different levels (species, genera, families; see Legatt and Iwama, 2003; Le Comber and Smith, 2004; Comai, 2005; Fontana et al., 2001).

2.3. Natural allopolyploidy and reproductive modes

Allopolyploidy arises by multiplication of chromosome sets resulting from intergeneric or interspecific hybridisations. Natural reproductive contacts between distantly related species in lower vertebrates may sometimes give rise to altered, but evolutionarily highly conserved, gametogenetic mechanisms in their progeny (Ráb et al., 2006). This is often associated with allopolyploidy and changes in reproductive modes, including parthenogenesis, gynogenesis and hybridogenesis, resulting in either clonal

(parthenogenesis and gynogenesis) or hemiclonal (hybridogenesis) inheritances (Schlupp, 2005). More than 90 cases of such hybrid complexes with altered modes of reproduction have been documented among lower vertebrates (Vrijenhoek et al., 1989; Alves et al., 2001). These phenomena are not rare, but asexual vertebrates are of a cryptic nature and genetic methods need to be used for their discovery (Ráb et al., 2006). In Europe, for example, there are several known hybrid asexual fish, including the cyprinids endemic Iberian minnow, *Iberocypris* (formerly *Squalius*) *alburnoides*, and Prussian carp, *Carassius gibelio*, an invasive Asian intruder, and cobitid spiny loaches of the genus *Cobitis* in the entire Europe north of the Mediterranean, plus several other suspicious cases (Ráb et al., 2006).

2.4. Natural allotriploids

Generally, spontaneous triploids are expected to be sterile due to interference with gametogenesis, resulting in vestigial or highly delayed gonadal development, or to be at least infertile due to random segregation of trivalents producing aneuploid gametes incapable of fertilisation. Accordingly, triploids would be regarded as dead ends in reproductive lineages. This, however, is not always the case. For instance, several natural diploid-polyploid hybrid complexes are known in fish, in which allotriploid females are fertile and reproduce gynogenetically from triploid eggs, *e.g.*, Prussian carp and *Cobitis* spp. (Cherfas, 1966; Kobayashi et al., 1970; Liu et al. 1978; Ueda and Ojima, 1978; Lusková et al., 2002; Vasiľev et al., 2003; Juchno and Boron, 2006). Alternatively, allotriploid females can produce eggs of different sizes and ploidies simultaneously, *e.g.* Iberian minnow and cyprinid or weather loach, *Misgurnus anguillicaudatus* (Alves et al.,

2001; Zhang et al.; 1998; Momotani et al., 2002; Alves et al., 2004; Oshima et al., 2005). Such features support the classification of these species as potentially cryptic complexes.

The fertility of allotriploid males in such complexes differs according to the species: males of Prussian carp are fertile (Shen et al., 1983), or capable of egg activation by motile aneuploid spermatozoa (Flajšhans et al., 2008). Males of the diploid-triploid hybrid complex of Iberian minnow produce unreduced diploid and triploid sperm (Alves et al., 1999; Sousa-Santos et al., 2007). Diploid-triploid mosaic males of cyprinid loach produced fully fertile unreduced diploid sperm with identical genotype (Morishima et al., 2004), while triploid males were found to be generally sterile, producing at best few haploid sperm (Oshima et al., 2005). The discovery of fertile natural triploids in the stone loach, *Noemacheilus barbatulus* (Collares-Pereira et al., 1995) suggests that this could be another cryptic species complex (Ráb et al., 2006).

2.5. Natural auto- and allotetraploidy

Autotetraploids occur through doubling of the diploid chromosome set within a species. Allotetraploids originate from hybridisation, usually between closely related species, whenever the chromosome complement derives from the sum of the diploid chromosomes sets of both species. Both auto- and allotetraploidy occur in nature (Gallardo et al., 1999). Spontaneous tetraploids were found, *e.g.*, in loaches (*Cobitis biwae*, *Misgurnus anquillicaudatus*) where viable tetraploid lines were developed (Arai, 2001; Yoshikawa et al., 2008), in Prussian carp (Flajšhans et al., 2008) and rarely also in the Iberian minnow (Alves et al., 1999). Some common aquaculture species or higher *taxa* are evolutionarily polyploid (sturgeons, *Acipenser sp*., common carp, *Cyprinus carpio*, crucian carp,

250 *Carassius auratus gibelio*) or derived from such polyploid ancestors (salmonids). The salmonids as a group underwent an autotetraploidisation process during their evolution
252 (Allendorf and Thorgaard, 1984).

254 Evidence from studies of artificially induced tetraploids suggests that natural auto- and allotetraploids may undergo an initial period of genomic instability, but later are able to
256 compete with their diploid relatives (Comai, 2005). Following autotetraploidy, chromosome sets tend to reduce genetic redundancy and revert gradually over long
258 periods towards diploidy (Comai, 2005).

260 **3. Artificial induction of polyploidy**

262 *3.1. Reasons for manipulation of ploidy in plants and terrestrial animals*

264 The generation of polyploids is by no means exclusive to fish and shellfish. Many plants used in modern agriculture are induced polyploids, selected to increase productivity, when
266 polyploidy is associated with greater cell size or disease resistance, and to produce seedless fruits from plants with uneven sets of chromosomes. Plants with altered ploidy
268 include: aneuploids (sugar cane), triploids (sugar beat, banana, apple, orange, lemon, or lime) tetraploids (cotton, potato, wheat for pasta, barley, leek, peanut, Arabica coffee, or
270 tobacco), hexaploids (wheat for bread or animal feed, garlic, kiwi, or plum), and octaploids (strawberry). Moreover, most species consumed as diploids originate from seed
272 productions that have used haploid steps in their genetic improvement (maize, cauliflower, rape, rice, asparagus, melon, or courgette).

274

Likewise, the advantages of sterilisation by castration is practiced in land animal production (bulls, pigs, poultry) to increase productivity and ameliorate meat quality; and in pets (dogs and cats) and horses or ponies to decrease their aggressiveness.

The induction of triploidy is an alternative approach to produce sterility in animals. Triploidy can be easily induced in some invertebrates and lower vertebrates but it is not clearly understood why is generally difficult or impossible to induce it in higher vertebrates. Hence, its domain of application is restricted to aquaculture and excludes land animal husbandry.

3.2. Principles of induction of triploidy in fish and shellfish

When mollusc eggs are released, they are arrested at the prophase or metaphase of Meiosis I (Colas and Dubê, 1998), while fish eggs are at the metaphase stage of Meiosis II on release (Colas and Dubê, 1998). Further development of the eggs is induced by the entry of the spermatozoon, leading to the resumption of meiosis I in shellfish or of meiosis II in fish. Physical or chemical shock applied during meiosis I or meiosis II can suppress cell division and prevent the extrusion of a polar body (either the first or the second in the case of shellfish but only the second in the case of fish), while allowing chromosomal division, thus producing triploids. Preventing the extrusion of the first (shellfish) or the second (fish and shellfish) polar body is thus key to the artificial induction of triploidy (Figs. 1 and 2).

Most cultured species of fish and shellfish release gametes into the water and can be readily triploidised. However, there are practical constraints with some shellfish such as the flat oysters and many crustaceans, where eggs are brooded in the mantle cavity or held

300 under the abdomen. Thus, the precise moment of fertilisation cannot be controlled. Among shrimps, for example, some species present internal embryo incubation and gametes are 302 not available for artificial fertilisation. Also, in some fish species such as tilapias, natural spawning hampers the efficiency of the treatment, thus making the induction of triploidy 304 more difficult.

306 The triploids depicted in Figs. 1 and 2 are sometimes referred to as maternal triploids because of the three sets of homologous chromosomes two are of maternal origin and one 308 of paternal origin. The production of paternal triploids, *i.e.*, with two sets of homologous chromosomes of paternal origin, is common in oysters when tetraploid males (producing 310 diploid sperm) are crossed with diploid females (section 7.2.3). This practice, however, is not common in fish, although possible. This has been achieved in the Buenos Aires tetra, 312 *Hemigrammus caudovittatus*, by incubating the semen with 2.5% polyethylene glycol to facilitate the entry of two sperm into an egg (David and Pandian, 2006); however, the 314 survival at hatching was more than 10-fold lower than maternal triploids obtained by either cold or heat shock.

316

Triploids can also be obtained by indirect methods based on interploid crossing, where 318 normal eggs are fertilised with the diploid sperm from a tetraploid male (*e.g.*, Wang et al., 2002; Nam and Kim, 2004; Francescon et al., 2004). Tetraploid fish can generally be 320 produced by inhibiting the first cell division of the zygote once the chromosomes have been duplicated shortly after fertilisation (Fig. 2). Viable tetraploid molluscs cannot yet be 322 produced by this method but are produced by a different method involving the use of eggs from triploids (Guo and Allen, 1994a) (section 5.3.2).

324

Allotriploids can be produced by natural crossing of two distantly related species or by backcrossing the fertile F_1 interspecific hybrids to one of the parental species (Arai, 1988, 2000; Benfey, 1989; Vrijenhoek et al., 1989; Pandian and Koteeswaran, 1998). Allotriploids induced by artificial interspecific hybridization followed by shock treatment to retain the second polar body can increase the viability with respect to diploid hybrids with poor vitality (Scheerer and Thorgaard, 1983). Closely related aquaculture species were sometimes intercrossed to generate allotriploids after triploidisation, as in flatfishes, salmonids and sparids (Purdom, 1972; Chevassus, 1979, 1983; Quillet et al., 1988; Gray et al., 1993; Gorshkov et al., 1998, 2002).

4. Effectiveness of current direct triploidisation techniques

4.1. Application of pressure and temperature shocks

Suppression of cell division can be achieved by several methods that include physical or chemical treatments. Physical treatments can be either pressure (Chourrout, 1984; Lou and Purdom, 1984; Benfey et al., 1988; Peruzzi and Chatain, 2000) or temperature shocks (Chourrout, 1984; Thorgaard et al., 1981), and the latter can be, in turn, either cold (*e.g.*, Colombo et al., 1995; Felip et al., 1997; Holmefjord and Refstie, 1997; Piferrer et al., 2000, 2003) or heat shocks (*e.g.*, Garrido-Ramos et al., 1996). Chemical treatments (*e.g.*, Thorgaard, 1983) use agents such as cytochalasin B, 6-dimethylaminopurine (6-DMAP) and caffeine that interfere with the microtubules during cell division, thus disrupting polar body extrusion (Beaumont and Fairbrother, 1991). Generally, physical treatments are the most successful and widely used to induce triploidy in fish (Teskeredžić et al., 1993; Haffray et al., 2007; Guoxiong et al., 1989; Johnson et al., 2004). Early trials with

350 shellfish generally revealed that physical treatments were less successful than chemical ones (Beaumont and Fairbrother 1991). More recent research demonstrates that heat shock
352 can be used to produce high proportions of triploids in shellfish (*e.g.,* Yang and Guo 2006) and this is valuable because cytochalasin B is not allowed for triploidy induction in the
354 European Union. Currently, triploid oysters in the United States of America (USA) and in Europe are almost exclusively produced commercially by mating tetraploid with diploid
356 oysters.

358 Pressure shocks consist of a transient, abrupt increase in hydrostatic pressure applied to fertilised eggs. The underlying mechanism of pressure-induced triploidisation has not been
360 thoroughly investigated and probably involves either an effect of pressure acting on the oolemma, literally resisting the extrusion of the second polar body, or an effect on the
362 meiotic spindle, or both.

364 A problem in the relevant literature is the use of different units of measurement when reporting the amount of hydrostatic pressure applied: atmospheres, bars, kg-force per
366 square centimetre and pounds per square inch. This variety hampers comparisons and makes it difficult to fully appreciate differences and similarities between species and
368 strains of fish. Here, we recommend for purposes of scientific reporting the use of the unit of pressure accepted in the International Systems of Units, the Pascal (Pa), and to express
370 the amount of pressure applied to fish eggs in Mega-Pascals (MPa). When conversions are made, it is interesting to observe that, despite enormous differences (~350-fold) in the
372 volume of fish eggs across species, from the small ~1 mm diameter of the pelagic eggs of many marine species (vol. ~0.5 µl), to the large ~7 mm diameter of the demersal eggs of
374 some salmonids (vol. ~180 µl), the optimal amount of pressure shock to prevent the

extrusion of the second polar body is quite similar, around 62 MPa in most cases (range: 376 58–85 MPa).

378 Since intensity is the second most important variable of pressure shock to induce triploidy (section 4.2), then a value of 62 MPa could be a good starting point when inducing 380 triploidy in a new species allowing the experimenter to concentrate on determining the best shock timing and shock duration. On the other hand, temperature shocks show more 382 variability. To start with, they can be either cold or heat shocks. Temperature shocks may prevent second polar body extrusion by altering development rates, disrupting the 384 microtubules of the meiotic spindle or indirectly through changes in cytoplasm density. Table 1 provides common values for timing, intensity and duration of shocks to induce 386 triploidy in both fish and shellfish.

388 Fish with large eggs display larger intrinsic variations to direct triploidisation treatment by temperature shocks. Pressure treatments seem to give results that are more reliable in these 390 cases, depending on the species. Cold shocks and pressure shocks are equally suitable for fish species with small eggs (carps, European sea bass, *Dicentrarchus labrax*, turbot, 392 gilthead sea bream, *Sparus aurata*, etc.) or sturgeons. However, pressure shocks are easier to apply in the case of floating eggs and large volumes of eggs in a commercial setting. 394 Sticky eggs (typical for most cyprinids or European catfish, *Silurus glanis*) should undergo a desticking procedure prior to the shock treatment, in order to prevent further incubation 396 problems.

398 *4.2. Importance of fine-tuned variables and egg quality to induced triploidy*

400 The major variables influencing the effectiveness of pressure and temperature shocks are, in order of decreasing importance, timing, intensity and duration of shock (Felip et al., 402 1997). To obtain 100% triploidy, application of a precise protocol is required. The critical values for each variable are species-specific and optimisation of all three is advisable to 404 obtain the highest triploid yield (Piferrer et al., 2000; 2003). Currently, triploidisation protocols are available for a considerable number of species of commercial interest farmed 406 in Europe (Felip et al., 2001a) but need to be optimised for others. Adapting these protocols to new species presents no major challenges. However, small changes in the 408 above variables may result in significant changes in triploid yields and therefore several trials may be required before the triploidisation conditions are optimized.

410

In fish, fertilisation and embryonic developmental rates are related to water temperature. 412 Thus, the optimum timing of a triploidisation shock depends on temperature of the water used for gamete activation, being especially critical for rapidly developing eggs of 414 warmwater species. The use of tau zero (τ_0), a unit of relative embryological age equivalent to the duration of one mitotic cycle during synchronous cell division, was 416 proposed in order to standardize shock timing (see Gomelsky, 2003). Once the species-specific duration of one mitotic cycle at particular temperatures is known, zygotic stages 418 when shock initiation is effective can be defined in τ_0 units (Cherfas et al., 1994; 1995b). This would allow others to recalculate the optimum start of a shock according to their 420 current temperature available for gamete activation and egg incubation. However, there is still a limited range of fish species for which the τ_0 is known, and thus many authors prefer 422 to use standardized incubation temperatures.

An adequate scaling-up of the method from laboratory to hatchery is a key step if triploidisation is to be applied at the large scale required for mass production and should be developed in collaboration with the aquaculture industry. Depending on the objective pursued, it is more or less important to achieve a 100% triploidisation rate.

As with normal aquaculture production, egg quality is important to optimize triploid larval yield. Kjørsvik et al. (1990) and Komen and Thorgaard (2007) concluded that egg quality is still a poorly understood complex phenomenon. Komen and Thorgaard (2007) also stated that some factors in eggs that can improve fertilisation and survival of fry, *e.g.*, fatty acid composition in marine species, may also affect survival after heat or pressure shocks. Other factors involve optimum timing of ovulation following hormonally-induced maturation or proper checking for ovulation in species where hormonal treatment cannot be reliably used, which could otherwise lead to ovulation of immature or over-ripe eggs. The consequences of egg overmaturation may lead to destabilisation of the meiotic spindle, as discussed above in Section 2.2.

Like the physical treatments used on fish, chemical treatments employed to induce triploidy in shellfish are highly sensitive to the concentration of the chemical, the start time of the shock, its duration, the temperature, and the quality of the eggs (Table 1) (Beaumont and Fairbrother, 1991).

4.3. Ploidy level determination

The experimental induction of polyploidy must be followed by an accurate determination of the expected ploidy level in the manipulated organisms. Methods to check the ploidy

level may be direct, such as karyotyping, measurement of DNA content, genotyping of microsatellite DNA markers, nucleolar-organising region (NOR) analysis, or indirect such as nuclear or cell size measurements (Fig. 3). Because triploids have an extra chromosome set and their nuclei and the cells themselves are larger than the equivalent nuclei or cells of diploids (Ihssen et al., 1990), a popular, low-cost, simple indirect method applied in fish is the measurement of the long axis of erythrocytes (Wolters et al., 1982; Benfey and Sutterlin, 1984a, b; Benfey, 1999, and others).

However, the precise determination of the ploidy level requires a direct method such as DNA content by flow cytometry, which is used by the animal production and food industry, and allows the analysis of several hundred individuals every day (Allen 1983; Lecommandeur et al., 1994). Sometimes the induction of triploidy results in mosaics (Arai, 2001; Teplitz et al., 1994) in which the ploidy level varies across tissues. In these cases it is important to verify that the germ cell precursors are triploid by using a direct method in order to ensure sterility. This is a potentially very important problem. Sterility would not be ensured if the mosaicism affects the gametes. Mosaic triploids should be suspected whenever there is egg release or milt can be stripped and found to be haploid by flow cytometry. The advantages and limitations of the various methods available to determine ploidy have been discussed by Harrell and Van Heukelem (1998), Linhart et al. (2001), Ocalewicz et al. (2006), and others.

5. Applicability of tetraploidy to generate auto- and allotriploids

5.1. Production and maintenance of artificial tetraploid broodstocks

Artificial tetraploidisation of a diploid species is theoretically possible through the suppression of the first cleavage but in practice this has proved difficult to achieve in a large number of fish species and, particularly, in shellfish. Thus, viable tetraploids have only been produced using this method in some fishes (see Table 2 and section 5.2.1). An alternative method using eggs from triploids (Guo and Allen, 1994a) has been successfully developed to produce tetraploid Pacific oysters for commercial use (section 5.2.2).

Two potential advantages of tetraploidy are overall increased heterozygosity, leading to heterosis (Diter et al., 1988), and gene redundancy, which masks recessive alleles (in gametes as well as zygotes) and provides evolutionary potential for diversification of gene function. Disadvantages of tetraploidy include changes in cell architecture that drive a decrease in cell numbers to maintain similar body size to diploids, especially in shellfish (section 5.2.2). Diploid spermatozoa from tetraploid fish may exhibit reduced fertility, as their enlarged heads have more difficulty in passing through the oocyte micropyle (Chourrout et al., 1986; Blanc et al., 1993). There can also be problems with the mechanics of the pairing and separation of chromosomal homologues during mitosis and meiosis that lead to aneuploidy (McCombie et al., 2005a). Regulatory changes in gene expression following tetraploidisation may result in epigenetic instability, because they are more likely to be deleterious than advantageous.

5.1.1. Fish

In general, tetraploids are difficult to produce. Yoshikawa et al. (2008) recently reviewed that viable mature and fertile tetraploids have been only obtained in rainbow trout, *Oncorhynchus mykiss*, blunt snout bream, *Megalobrama amblycephala,* and mud loach.

Misgurnus mizolepis, but not in other species of aquaculture importance. As illustrated in Fig. 2, autotetraploids are produced in fish by suppression of the first cleavage division using chemical or physical (heat/cold shock, pressure shock) methods. This method was successfully developed initially in rainbow trout (Thorgaard et al., 1981; Chourrout, 1982; 1984; Chourrout et al., 1986). Tetraploid fish were produced also in channel catfish, *Ictalurus punctatus* (Bidwell et al., 1985), tilapias of the genus *Oreochromis* (reviewed by Mair, 1993), grass carp, *Ctenopharyngodon idella* (Zhang et al., 1993; Cassani et al., 1994, common carp (review by Gomelsky, 2003), tench, *Tinca tinca* (Flajšhans et al., 1993), Indian carps, *Labeo rofita* and *Catla catla* (Sarangi and Mandal, 1994), and yellow perch, *Perca flavescens* (Malison et al., 1993a) (see also Table 2). In many cases, low yields of larvae, which either did not survive to the fingerling stage or died later on, were reported.

Some problems have been identified in mature tetraploids, such as the lower fertilising ability of tetraploid males (Chourrout et al., 1986) —which could be solved by the selection of tetraploid males with smaller spermatozoa head size (Blanc et al., 1993)—, low frequency of unbalanced or unreduced ova produced by tetraploid females, spontaneous androgenesis (Chourrout and Nakayama, 1987), and diploid-tetraploid mosaicism in different organs (Yamaki and Arai, 2000). These observations imply that a strict ploidy and pedigree control (using DNA fingerprinting methods) is required for breeders to avoid contamination of the tetraploid stock by unwanted genotypes. Interestingly, a study on rainbow trout suggested that, even though it is the first cleavage division that is targeted, the actual chromosome doubling process may only take place during the second cleavage division (Zhang and Onozato, 2004), explaining the high risk of diploid/tetraploid mosaicism.

If irradiated or DNA-denatured sperm is used to activate the eggs (not true fertilisation), targeting the first cleavage division produces diploid gynogens. In this regard, recently the importance of observed broodstock-level changes in time to the first cleavage (*i.e.*, first cleavage interval [FCI], a crude variation of τ_0; see section 4.2) in rainbow trout can be important because Hershberger and Hostuttler (2008) demonstrated that the most successful protocol to block the first cleavage in two different populations involved treatment starting at 62–65% of the FCI. Therefore, prior FCI analysis will assist tetraploidy induction.

5.1.2. Shellfish

5.1.2.1. Inapplicability of suppression of the first cleavage

Direct induction of tetraploidy has proved difficult in shellfish. Efforts to produce viable adult tetraploid bivalves and shrimp by suppression of the first cleavage have consistently failed (Allen et al., 1994; Yang et al., 2000; Peruzzi and Guo, 2002; Yang and Guo, 2004, 2006; Sellars et al., 2006a). In spite of the fact that significant proportions of tetraploid embryos of bivalves can be detected following suppression of the first cleavage, and that some of these may develop through to the veliger larval stage, none survive beyond the spat stage. Indeed, Yang and Guo (2006) followed the development of a cohort of dwarf surfclam, *Mulinia lateralis,* larvae that had resulted from induction of tetraploidy at first cleavage. Both diploid and tetraploid embryos were present at 24 h, but by day 6 the normal D-shaped veliger larvae were all diploid, while the unshelled, abnormal larvae

were a mixture of diploid and tetraploid. This confirmed that the tetraploid larvae all underwent abnormal development.

The most likely cause of this lack of viability is the “cell-number deficiency” hypothesis (Guo, 1991, Guo and Allen 1994b), which stems from the fact that a tetraploid cell is expected to contain twice as much nuclear material as a diploid cell. Therefore, tetraploid cells will be larger than diploid cells and there is a contradiction during development between numbers of cells and the size of the organism. Thus, assuming the overall organism size stays similar to diploids, fewer cells will be present.

In molluscs, key decisions during early development seem to be made very early when there are only a few cells in the embryo. The approximate cell number at which specification of parts can be detected by isolation experiments is 2 cells in bivalves but between 32 and 64 cells in vertebrates (Slack, 1983). Cleavage of a normal-volume bivalve egg containing a large tetraploid nucleus is more likely to lead to cell-number deficiency compared to fish eggs, where the later development of morphogenic controls allow blastomeres to interact during segmentation and to compensate for any cell-number deficiency.

A further potential complication in the suppression of first cleavage in bivalve molluscs is the presence of a polar lobe, a cytoplasm-filled sac that is extruded and then reabsorbed during first and second cleavages, respectively (Slack, 1983). The polar lobe is extruded from the so-called CD blastomere at first cleavage, a process that is likely to be disrupted by artificial suppression of the first division. Because this cytoplasm is finally reabsorbed into the D blastomere after the second cleavage, and because some aspects of embryo

determinism are already present in the cytoplasm at this stage, it seems possible that disruption of normal polar lobe behaviour could have a role in the abnormal development of tetraploid embryos.

5.1.2.2. Alternative techniques to produce tetraploid bivalves

Guo (1991) proposed that the problem of cell-number deficiency in tetraploid embryos might be circumvented by an increase in the egg volume. He tried zygote-zygote fusion in the Pacific oyster but without success. He also attempted to select diploid females with large eggs, but egg size variance is low in this species, so he found this difficult. Guo and Allen (1994c) discovered that eggs from triploid oysters (some triploid oysters do produce a few eggs) were significantly larger (15% increase in diameter; 54% increase in volume) than normal eggs from diploid oysters and that this offered an alternative route to obtain tetraploid oysters. Finally, Guo and Allen (1994a) published a method to make viable tetraploid Pacific oysters by crossing eggs from a triploid with spermatozoa from a diploid and then suppressing the extrusion of the first polar body (Fig. 4). This method is now patented in the USA with licenses for EU use (USA Patent #5824841; 20/10/1998).

This method is limited by two main factors. Firstly, fecundity in triploid oysters is extremely low and only small numbers of eggs can normally be obtained from triploid oysters. Secondly, only a small proportion of the offspring is actually tetraploid. In the Guo and Allen's (1994a) study, only 0.074% of the treated eggs produced tetraploids (identified at the spat stage), although this represented 2,400 spat. By three months old, when oysters were 1–4 cm in shell length, 66% of them were identified as tetraploid by chromosome counting.

598

Eudeline et al. (2000a) found it difficult to repeat Guo and Allen's (1994a) method on
600 Pacific oyster and explored variations in shock timing and duration to block the release of the first polar body. They demonstrated that this process is delayed in eggs from triploid
602 oysters as compared to those from diploid oysters, and that this delay varied among individual females (Eudeline et al., 2000b). Best results were obtained by monitoring the
604 onset of meiotic resumption in a sub-sample of eggs to identify the precise timing for the application of treatment. Subsequent work has successfully utilised this method on several
606 other bivalves (Table 2), but some species (*e.g., M. galloprovincialis*, *M. arenaria*, and *S. commercialis*) do not produce suitable eggs from female triploids (Yang and Guo, 2006).

608

Alternative methods for tetraploidy induction in bivalves have also been explored.
610 Although expected to produce only triploids, suppression of the expulsion of the first or second polar body in bivalves can sometimes produce a small percentage of tetraploid
612 embryos (*e.g.*, Diter and Dufy, 1990; Yang et al., 2000; Peruzzi and Guo, 2002; Yang and Guo, 2004). The mechanism for this outcome is not fully understood. However, as with
614 the tetraploid embryos achieved by suppression of the first cleavage, very few, if any, tetraploid embryos from the first or second polar body suppression survive beyond the
616 larval stage. Scarpa et al. (1993) used cytochalasin B to suppress both the first and second polar bodies in eggs of the mussel *M. galloprovincialis* fertilised with normal sperm and
618 obtained varying ploidies from diploid to decaploid. A small number of one-month-old tetraploid spat were produced, but there is no record that these were subsequently grown to
620 maturity.

One approach that can introduce new genetic material into oyster tetraploid broodstock without going through the Guo and Allen (1994a) triploid egg method has been demonstrated by McCombie et al. (2005b). Eggs from diploids are fertilised with diploid sperm from tetraploids and, afterwards, the second polar body is suppressed to produce tetraploid offspring. Although other types of modified ploidy were produced, some tetraploids were identified at six months following this method, which confers the introduction of a new diploid genome.

5.2. Use of tetraploids in triploid production

5.2.1. Fish

In rainbow trout, the production of unreduced eggs and enlarged spermatozoa in tetraploids limits their value as a tool to produce triploids. Chourrout et al. (1986) and Blanc et al. (1987) compared triploid rainbow trout produced by mating tetraploid males with diploid females against triploids obtained by heat shock from diploid females to inhibit second polar body extrusion. A second generation of tetraploids was produced by heat shock of diploid females fertilised by tetraploid sires. Second generation tetraploids were also inferior in growth to other ploidy types, but grew better than first-generation tetraploids.

The mud loach seems to tolerate polyploidy better. Nam and Kim (2004) found that 6% of tetraploid males permanently produced diploid sperm, while the rest released haploid or aneuploid sperm. By crossing normal females with tetraploid males releasing diploid sperm they achieved 100% triploids. The origin of the variation in ploidy level in the

sperm of tetraploid males is not known, but indicates that sperm ploidy must be
648 preliminarily checked for successful triploidisation.

650 It should be noted that, in auto- or allotriploids obtained by blocking the extrusion of the second polar body, the double maternal chromosome complement derives from sister
652 chromatids and is necessarily homozygous, except at those loci affected by recombination at Meiosis I. In triploid fish resulting from interploid crossing, the tetraploid mother (or
654 father) transmits a pair of homologous chromosomes, which have passed through Meiosis I and presumably have undergone recombination events. Their heterozygosity may be
656 decreased or increased by crossing-over., Thus, the differences between triploids obtained by crossing diploids with tetraploids and those induced by physical or chemical treatment
658 include the presence or absence of treatment, the different genetics and the general performance.

660

5.2.2. Shellfish

662

The principal commercial value of oyster tetraploids is their use in crossing with diploids
664 to produce 100% triploids. Ten years later than in fish, Guo et al. (1996) were able to confirm the usefulness of tetraploid oysters in the production of triploids. They showed
666 that both sexes of tetraploid Pacific oyster produced by the Guo and Allen (1994a) method are fertile and that reciprocal crosses between tetraploids and diploids give rise to 100%
668 triploids (Guo et al., 1996). Dong et al. (2005) showed that sperm from tetraploid oysters can be collected and cryopreserved for later use in the production of triploids.

670

Tetraploid Pacific oyster have also since been used to make triploid hybrids with the
672 Suminoe oyster, *Crassostrea ariakensis* (Que and Allen, 2002). Hybrid triploid spat were produced using diploid spermatozoa from a male tetraploid Pacific oyster but no triploid
674 larvae survived from the reciprocal cross (diploid eggs from tetraploid *C. gigas* x sperm from *C. ariakensis*).

676

5.3. Performance of tetraploids and maintenance of tetraploid stocks

678

5.3.1. Fish

680

Chourrout et al. (1986) found very low rainbow trout tetraploid larval and juvenile
682 survival relative to triploid and diploid progenies from the same parents. By six months old, survival had stabilised in the tetraploid group and tetraploidy was confirmed by
684 erythrocyte size. Many male rainbow trout tetraploids matured in two or three years and produced milt capable of fertilising eggs of diploid females. Sperm of tetraploid rainbow
686 trout was also successfully used in androgenesis (Thorgaard et al., 1990) to replace shock-mediated restoration of diploidy contributing to enhanced mortality of androgenic diploid
688 embryos. However, in the mud loach not all tetraploid males produced diploid spermatozoa (Nam and Kim, 2004). Of 48 males tested, twelve had significantly reduced
690 gonad development, 26 had normal gonads with haploid sperm and only three had diploid sperm. The remainders were mosaics. Nevertheless, each individual retained the same
692 ploidy of the sperm produced over time (Nam and Kim, 2004).

694 *5.3.2. Shellfish*

696 In shellfish, tetraploid Pacific oysters have been produced (Guo and Allen 1994a, Fig 4) and these tetraploids have fertile diploid eggs and spermatozoa that allow reciprocal
698 crosses between tetraploids and diploids to produce 100% triploids (Guo et al., 1996). These authors produced a second generation of tetraploid Pacific oyster by direct crossing
700 between tetraploid individuals but the tetraploid spat grew slower than triploids produced by tetraploid x diploid mating and their survival to the spat stage was only 0.1% of control
702 diploids. Some, or most, of this poor performance could have been due to inbreeding, because the parental tetraploids were siblings.

704

However, Guo and Allen (1997) investigated maturity in auto-tetraploid Pacific oysters
706 and demonstrated that they became mature at one year old, had a 1:1 sex ratio and produced balanced diploid gametes, with the two extra chromosomes in the quadrivalents
708 co-segregating to opposite poles of the first meiotic spindle. This is in contrast to triploid oysters where the extra chromosomes in the trivalent separated at random during the
710 anaphase of meiosis I.

712 To reduce inbreeding as much as possible, tetraploid broodstock need to be genetically unrelated to one another, but this creates a problem in oysters because of the rarity of
714 triploids that have suitably viable eggs and the difficulty of optimizing the Guo and Allen (1994a) method (Eudeline et al., 2000a).

716

5.4. Potential genetic impact of tetraploid escapees

718 Because artificially produced allo- and autotetraploid fish and shellfish have been demonstrated to be fertile under laboratory or hatchery conditions, their release into the
720 environment poses a major ecological and biodiversity risk. A classic example of the

potential genetic impact of novel fertile tetraploids in the maritime environment is the case of the sea grasses of the genus *Spartina*. The smooth cord grass, *Spartina alterniflora*, was introduced from the east coast of North America to Southampton waters in the late 1800s where it hybridised with the local cord grass, *Spartina maritima*, to produce a sterile hybrid, *S. townsendii*. Later, this hybrid underwent amphidiploidy to become a tetraploid species, *S. anglica*, which exhibited rapid growth, high fecundity and aggressive colonization on mud flats (Hubbard and Stebbings, 1967). The ecological effects are significant and include the rapid colonization of extensive mud flats, which has reduced habitat for bird feeding and roosting, the virtual replacement of *S. maritima* by *S. anglica*, and the reduced biodiversity where *S. anglica* becomes the monotypic climax community (Davidson et al., 1991).

Thus, because there is such a significant risk of potential genetic and environmental impacts following the escape of tetraploids, appropriate measures must be taken, and it is essential that tetraploid fish or shellfish broodstocks —whether used for commercial purpose or experimental work— be held in quarantine. This will prevent the accidental release of gametes or the escape of tetraploid larvae, juveniles or adults into the environment. Only small broodstocks should be kept and these must be properly managed and monitored by Government agencies or Government-licensed companies. In Europe, in order to anticipate or prevent potential negative interactions with local species, the European Council in its Council Regulation concerning “Use of alien and locally absent species in aquaculture” considers fertile polyploids such as tetraploids as exotic species, and thus it is necessary to follow the same precautionary procedures: official demand to the Member State, environmental risk assessment and quarantine for their rearing. For example, in France tetraploid oyster broodstocks are held by IFREMER in closed

recirculated systems equipped with water treatment systems to prevent dissemination of gametes or larvae.

6. Performance capacity of triploids with respect to diploids

6.1. Overview of consequences of induced triploidy

Performance of triploids is species specific and is well documented in laboratory-scale but far less so at production scale. The performance of triploids has been evaluated in freshwater and anadromous fishes (Thorgaard, 1983; Benfey, 1991, 1999; Gomelsky, 2003; Mair, 1993; Ihssen et al., 1990; Arai, 2001), marine fishes (Felip et al., 2001a), and shellfish (Beaumont and Fairbrother, 1991). Very few results are reported in crustaceans because triploidisation has not been performed in most of cultured species due to specific reproductive aspects (section 3.2). Nevertheless, it should be borne in mind that when comparing the performance of triploids *vs.* diploids, the existence of some treatment x family interactions do exist (*e.g.*, Johnson et al., 2004). Thus, caution should be taken when interpreting limited data sets or when generalizing.

Several aspects of the physiology and behaviour of triploid fishes were reviewed by Benfey (1999) and research is updated below for major aspects (survival. growth, behaviour, reproduction). However, differences between diploids and triploids have been investigated also for other traits, *e.g.*, haematology (Benfey, 1999; Ballarin et al. 2004; Peruzzi et al., 2005; Cal et al., 2005), carcass quality (*e.g.*, Peruzzi et al., 2004; Buchtová et al., 2005; Segato et al., 2007; Poontawee et al., 2007; Werner et al., 2008), flesh quality

(Bjornevik et al., 2004; Buchtova et al., 2004, Poontawee et al., 2007), and immunology (Budiño et al., 2006; Maxime, 2008).

6.2. Survival

6.2.1. Fish

Numerous studies showed lower early survival of triploids relative to diploids due to lowered viability of eggs, developing embryos and hatched larvae up to the first feeding stage, as reviewed, *e.g.*, by Chourrout (1988), Ihssen et al. (1990), Thorgaard et al. (1992), Pandian and Koteesvaran (1998), Benfey (1999), Arai (2001), Felip et al. (2001a), Hulata (2001), Gomelsky (2003), Tiwary et al. (2004), and Maxime (2008).

If shock treatment is not 100% effective, cohorts of putative triploids will often contain many diploids that have failed to respond to the shock treatment. Such “shocked” diploids provide useful controls for the effect of shock treatment alone rather than the condition of triploidy. In performance comparisons between shocked triploids, obtained by direct induction, and interploid triploids, obtained by crossing tetraploids with diploids, the survival of interploids was enhanced with respect to shocked triploids and was similar to that of diploids (Chourrout et al., 1986; Blanc et al., 1987; Myers, 1991; Myers and Hersberger, 1991). Further, Cherfas et al. (1994) showed that shocked diploids and triploids presented the same survival during early stages, but had lower survival than the unshocked diploid controls. This suggests that the induction shock is the main factor responsible for early depressed survival, whereas the triploidy status itself may be the cause of reduced survival later on. In immature European sea bass, during grow out

between 10 and 100 g, both unshocked and shocked diploids survived equally better than
796 triploids (J.-C. Falguière, unpublished data).

798 During separate grow out in juvenile fish, survival of triploids tends to be similar to, or lower than, that of diploids, at least under optimal conditions, as observed in rainbow trout
800 (Quillet et al., 1988; Ojolick et al., 1995; Sheehan et al., 1999), Atlantic salmon, *Salmo salar* (McGeachy et al., 1995; Oppedal et al., 2002), tench (Flajšhans et al., 1993, 2004),
802 red sea bream, *Pagrus major* (Sugama et al., 1992), gilthead sea bream (Haffray et al., 2005), and European sea bass (Felip et al., 1999).

804

On the other hand, when reared together (a.k.a. common garden), lower survival of
806 triploids with respect to diploids during the grow out phase has been repeatedly reported. In coho salmon, *Oncorhynchus kisutch*, Utter et al. (1983) showed that a stock composed
808 of 85% of triploids, decreased to only 19% at 17 months. Similar observations were made on Atlantic salmon (Galbreath et al., 1994), common carp (Cherfas et al., 1994), rainbow
810 trout (Thorgaard et al., 1982), brown trout (Bonnet et al., 1999), and European catfish (Linhart et al., 2001). Nevertheless, other studies in Atlantic salmon (McGeachy et al.,
812 1995) and tench (Flajšhans et al., 1993) did not find differences in survival due to ploidy levels. “In summary, in most cases, mixed grow out result in differences in survival of
814 diploids and triploids (Table 4)”.

816 There are few references that report on the survival rates of triploids as adults. Triploid rainbow trout reared in seawater show a better survival due to mass mortalities of diploid
818 females (Lincoln and Scott, 1984; Quillet et al., 1987). Triploid ayu, *Plecoglossus altivelis*, survive while diploids die after spawning (Ueno et al., 1986), but adult triploid

and diploid gilthead sea bream have similar survival rates (Haffray et al., 2005). Adult triploid turbot had 8% better survival than diploids due to the lack of postspawning mortality in triploids (Cal et al., 2006).

Triploids with higher genomic heterozygosity than diploids are expected to be more viable, and thus the cause of the lower survival of triploids sometimes observed during the grow out phase has not been elucidated. In this regard, the bigger size but lower number of their erythrocytes and a reduction in cell surface available for gas exchange may depress aerobic capacity at low oxygen concentrations in triploids (Benfey, 1999). In Atlantic salmon, McCarthy et al., (1996) suggested a lower adaptation capacity of triploids to seawater cages even if the difference in growth rate was not significant. Sadler et al. (2000a,b) did not find any difference of oxygen carrying capacity and haematological response to confinement stress nor in plasma cortisol or lactate at different juvenile stages before transfer to seawater. A significant genetic component of survival after the transfer in seawater independent of family growth was observed in diploid and triploid brown trout, *Salmo trutta* (Bonnet et al., 1999). This indicates that a genetic basis for survival exists and can be used to improve survival in seawater.

Measurements of aerobic capacity revealed no deficiency in triploid salmonids (Ojolick et al., 1995; Stillwell and Benfey, 1997), and tests on exhaustive exercise in brook trout, *Salvelinus fontinalis*, triploids showed that aerobic and anaerobic capacities of triploids were only compromised relative to diploids at high temperatures (Hyndman et al., 2003a, 2003b). Further, forced swimming in triploid Atlantic salmon demonstrated no difference in their aerobic capacity compared with diploids, but anaerobic capabilities were affected (Cotterell and Wardle, 2004).

The cardiorespiratory performance of triploid brown trout in seawater was not different from diploids at 18°C, but a plateau in maximum cardiac performance was observed between 14 and 18°C, which could be associated with observed mortality at 18°C (Altimiras et al., 2002; Mercier et al., 2002). This difference has been recently confirmed by Atkins and Benfey (2008), reporting that triploids of brook trout and Atlantic salmon have lower thermal optima than diploids, something that can explain prior observations of high mortality of triploids at chronically elevated, but sub-lethal, rearing temperatures. However, Galbreath et al., (2006) could not confirm these observations in rainbow trout and brook trout reared in fresh water using other endpoints, such as the evaluation of the time in which each individual fish reached loss of equilibrium in the water column (*i.e.*, turned on the back side) during regular increase (+ 2°C/day) of temperature

The gill lamellae of triploid tench exhibit a lower blood and water diffusion distance (thinner respiratory epithelium) than in diploids that could be a potential adaptation to decreased aerobic capacity (Flajšhans and Piačková, 2006). In shi drum, *Umbrina cirrosa*, triploids, Ballarin et al (2004) observed a lower concentration of circulating blood cells with a lower surface/volume ratio and suggested that this could be a disadvantage in stress conditions. These studies support the idea that the influence of triploidy on oxygen transport might only be manifested under adverse conditions (Bernier et al., 2004).

Maxime (2008) concluded that the magnitude and dynamics of physiological responses to acute stress were alike in triploids and diploids. Triploid and diploid brook trout and rainbow trout subjected to handling stress did not reveal differences in haematocrit, plasma cortisol and glucose profiles (Biron and Benfey, 1994; Benfey and Biron, 2000;

Legatt et al., 2006). Recent trials with three hatchery rainbow trout stocks also failed to show differences between ploidy levels in mortality with stocking (pH, temperature) or handling stressors (Wagner et al., 2006).

In summary, the temperature or pressure shocks used to produce triploids cause a whole range of undesirable effects that reduce survival especially during early development. In addition, the triploid condition could be responsible for the reduced survival (and growth) at later stages, particularly when environmental conditions are not optimal.

6.2.2. Shellfish

A similar situation to fish is observed in shellfish, where survival through the early embryo, trochophore and early veliger larval stages is often compromised by the triploidy induction treatment employed (*e.g.*, bivalves: Beaumont and Fairbrother, 1991; Nell, 2002; abalone: Zhang et al., 1998; Maldonado et al., 2001; Norris and Preston, 2003; Li et al., 2007; shrimp: Sellars et al., 2006b; Xiang et al., 2006).

The immune system is important for the survival of organisms and there have been studies on the comparative immunology of diploid and triploid oysters (Nell, 2002; Gagnaire et al., 2006; Duchemin et al., 2007). The immune system in bivalves relies partly on the number and type of circulating haemocytes (Cheng, 1996) and haemocyte abundance can vary throughout the annual reproductive cycle. During periods of low counts of circulating haemocytes, such as during gamete atresia, breakdown and resorption, bivalves are less able to resist bacterial or other disease challenge. Because of the different patterns of gametogenesis in triploid and diploid bivalves, their survival (following disease, pollution or stress challenge) is predicted to be different in different situations and for different

896 species (Nell, 2002). Indeed, improved stress resistance of triploid Pacific oyster compared with diploids is borne out by studies on their susceptibility to Summer Mortality
898 in the Marennes-Oleron region of France (Gagnaire et al., 2006). In contrast, Duchemin et al. (2007) did not find significant differences between the immunological health of diploid
900 and triploid Pacific oyster sourced from different areas of France but grown out in Brittany. It is possible that these conflicting results relate to the genetic background
902 (hatchery *vs.* wild) or the different geographic sources of the oysters used.

904 In the USA, research has been conducted into the possible introduction of the Suminoe oyster to rehabilitate the fishery of American or eastern oyster, *Crassostrea virginica*, and
906 to promote oyster reefs in Chesapeake Bay. Because the Suminoe oyster is not native to the region, experiments have deployed 100% triploids produced by mating tetraploids with
908 diploids (Guo and Allen 1994a; Guo et al., 1996). In a field comparison between triploid American oyster and triploid Suminoe oyster juveniles (but without diploid controls of
910 either species), Paynter et al. (2008) found no significant mortalities in either group.

912 *6.3. Growth*

914 *6.3.1. General considerations*

916 The growth of triploids *vs.* diploids, specifically in fish, has generated some confusion. Theoretically, triploids could grow faster than diploids for three main reasons: the cell size
918 "gigantism" effect, the possession of higher overall genomic heterozygosity and the diversion of energy from gonadal to somatic growth. The first two effects should operate
920 throughout the life of the triploids, while the third one is restricted to the adult stage.

First, the concept of gigantism in polyploids (at least in shellfish, Guo and Allen 1994a) is based on the idea that because the nucleus of each somatic cell must contain a larger volume of chromosomes (1.5 times more in the case of triploids *vs.* diploids) this will mean that cell size is increased. Assuming an equal number of cell divisions takes place in the production of a body tissue, then that tissue should occupy a larger volume in a triploid compared with a diploid. Because body size is correlated with cell size in some organisms, (Ihssen et al., 1990), it could be expected that triploid organisms would grow faster and reach larger ultimate sizes than diploids.

However, it was recognized a long time ago in experiments with amphibians that polyploids had bigger cells than diploids but were not significantly larger (Fishberg, 1944; Fankhauser, 1945), so this is an unlikely explanation (Swarup,1959). Indeed, it has been repeatedly demonstrated in fish that triploid cells are bigger, but triploids themselves neither reach larger ultimate sizes, nor grow faster than diploids (Ihssen et al., 1990; Pandian and Koteeswaran, 1998). Benfey (1999) reviewed these findings showing that increased cell sizes led to decreased cell numbers in different fish organs, such as brain, retina, epithelia, cartilage, muscle, liver, kidney, testes, ovaries and blood count, and concluded that the increased cell size did not appear to confer any growth advantage to triploids due to the concomitant decrease in cell number.

Second, an overall increase in genomic heterozygosity is expected in triploids relative to diploids because at every polymorphic locus there is the possibility of triploid individuals coding for three different alleles. Theoretically, triploids should exhibit higher constitutive developmental rates but evidence of this is inconclusive (Leary et al., 1985). The

946 assumption is made that individuals that possess three alleles at a locus (triploid triple heterozygotes, *e.g.*, such as *abc*) have a fitness advantage over individuals that possess two
948 alleles (diploid heterozygotes, *e.g.*, such as *ab, cb,* etc.). It is the same assumption that is made when comparing two-allele heterozygotes with homozygotes, but there is an
950 important distinction between them. The inbreeding effect, where homozygosity at coding loci is linked to deleterious recessives at linked loci, is not involved in the comparison
952 between the possession of two or three alleles at a locus. Locus-specific heterozygosity in induced triploid fish is complicated by whether the locus has been affected by
954 recombination or not during meiosis of the egg from which that triploid developed (Allendorf and Leary, 1984; Beaumont and Fairbrother 1991; Beaumont, 2000). In
956 molluscs, an additional variable is created depending on whether meiosis I or meiosis II is targeted (Beaumont and Fairbrother, 1991; Beaumont et al., 1995; Beaumont, 2000;
958 Beaumont and Hoare, 2003). Further, the advantage of higher heterozygosity of triploids needs to be estimated without the bias induced by triploidisation treatment and hence
960 crosses between tetraploids and diploids should be used for this purposes, as done in oysters.

962

Third, the sterility of triploids, which causes more energy to be available for somatic
964 growth since requirements for gametogenesis are totally (females) or partially (males) reduced, is supposed to produce a significant enhancement in the growth of triploids when
966 their diploid counterparts become mature. Table 3 summarizes the growth performance of triploids for the main cultured species of fishes, shellfish and crustaceans. During the
968 juvenile and immature adult phase, triploid fishes usually grow equal or less than diploids, depending on the species and environmental conditions. In shellfish, triploids generally
970 have a similar growth to diploids as juveniles. This suggests that triploids do not have an

intrinsic higher growth rate than diploids before maturation (Sheehan et al., 1999). On the other hand, in most cases growth is enhanced after maturation, especially in shellfish. Sometimes, triploid fish tend to divert excess energy saved from impaired reproduction into fat deposits rather than into the growth of lean mass. In such cases altering the nutritional regime is a possible approach to address the problem.

6.3.2. Fish

In Atlantic salmon, Benfey and Sutterlin (1984b), Quillet and Gaignon (1990), Jungalwalla (1991), Galbreath et al. (1994), O'Flynn et al. (1997), and Cotter et al. (2002) showed that triploids or all-female diploids do not present significant growth differences in freshwater when the two genotypes are grown in separate tanks, at least until 9 months of age. This is in agreement with results obtained in rainbow trout reared under appropriate environmental conditions (Sheehan et al., 1999; Legatt et al., 2006; Wagner et al., 2006) or in Arctic charr, *Salvelinus alpinus* (Gillet et al., 2001). Further, Quillet and Gaignon (1990) and Cotter et al. (2002) in Atlantic salmon, and Taylor et al., (2007) in rainbow trout, did not observe significant differences in smolting rates. In seawater cages, Jungalwalla (1991), Galbreath and Thorgaard (1995), O'Flynn et al. (1997) and Cotter et al. (2002) observed that triploid Atlantic salmon had the same growth rate as diploids until the onset of sexual maturation in the spring. Oppedal et al. (2002), however, found that triploids grew to commercial size faster than diploids in seawater tanks under natural or continuous photoperiod.

Adult triploids in many fish and shellfish species clearly grow faster than diploids and this must therefore be mainly due to the redirection of energy from gametogenesis to somatic

996 growth. This is usually encountered in adult triploid freshwater fishes, such as salmonids (except coho salmon), Nile tilapia, *Oreochromis niloticus*, catfishes (except African 998 catfish, *Clarias gariepinus*), tench, perch and cyprinid loach but it is less commonly detected in marine fishes, except turbot and flatfish allotriploids. Nevertheless, in some 1000 species such as the European sea bass or the gilthead sea bream, where sexual maturation depresses growth (Zanuy et al., 2001), triploids do not grow more, even when their diploid 1002 counterparts mature (Felip et al., 2001b; Haffray et al., 2005). Triploids may also have altered sex ratios due to imbalances in epistatic and autosomal sex determining factors 1004 (Devlin and Nagahama, 2002). This seems to be the case with turbot, where, by virtue of having sex ratios skewed in favour of females (the larger gender), triploid stocks attain a 1006 higher biomass at a given age than diploids (Cal et al., 2006). In hermaphrodite protandrous sparids, triploids also first develop as males (Kitimura et al., 1991; Haffray et 1008 al., 2005) and their growth is the same as of diploids during the male phase but is reduced once diploids initiate sex-change (Arakawa et al., 1987 Sugama et al., 1992; Haffray et al., 1010 2005).

1012 Shocked diploids grew faster than shocked triploids in grass carp (Cassani and Caton, 1986), common carp (Cherfas et al., 1994) and rainbow trout (Thorgaard et al., 1982). In 1014 the European sea bass, both shocked and control mature diploids grew faster than triploids (J.-C. Falguière, unpublished data), highlighting the negative effect of triploidy *per se* on 1016 adult growth.

1018 In some studies, triploids and diploids were reared together either because of unreliable induction protocols or for easier management practices. In such common garden 1020 experiments immature triploid fish frequently grow less well than diploids, although

differences in growth rate are not necessarily evident when triploids and diploids are reared separately (Table 4; Cassani and Caton, 1986; Lincoln and Bye, 1987; Quillet and Gaignon, 1990; Galbreath et al., 1994). This phenomenon has been observed in commercial culture of rainbow trout, (Galbreath et al., 1994) but not in laboratory experiments (Thorgaard et al., 1982); Thorgaard, 1986). Conflicting data about triploid growth relative to diploids in communal culture are known for coho salmon (Utter et al., 1983; Johnson et al., 1986) and African catfish (Henken et al., 1987). Although the factors involved in the competition between diploids and triploids are not clearly elucidated, they seem to be related to rearing conditions and may be species specific.

Sub-optimal rearing conditions can lead to poorer performances of triploids. Examples include defective adjustment to seawater culture in salmonids (Johnson et al., 1986; Quillet et al., 1987; Quillet and Gaignon, 1990; Galbreath and Thorgaard, 1995; Ojolick et al., 1995; Chevassus, 1998; Bonnet et al., 1999), high rearing temperatures in rainbow trout (Ojolick et al., 1995), grow out at high stocking density and communal culture in grass carp (Cassani and Caton, 1986) and low level of dissolved oxygen in salmonids (Quillet at al, 1987; Quillet and Gaignon, 1990; Johnstone et al., 1991; Ojolick et al., 1995; Stillwell and Benfey, 1997; Bernier et al., 2004) and European sea bass (J.-C. Falguière, unpublished data). Maxime (2008) concluded that triploids are especially affected by frequent occurrence of poor water quality in rearing ponds and that they are not able to cope well with such sources of chronic stress. The possible reasons for the greater susceptibility of triploids to adverse conditions are discussed in section 6.2.1.

6.3.3. Shellfish

In shellfish, the growth of adult triploids is generally enhanced relative to diploids in all species reviewed (Table 3), including oysters (Nell 2002), other bivalves (Beaumont and Fairbrother, 1991) and shrimp (Xiang et al., 2006). However, the growth advantage of triploids is not always clear (Ekaratne and Davenport, 1993) and may be affected by food availability (Racotta et al., 2008). For triploid Pacific oysters, increased growth is only one aspect of their value to aquaculture because the lack of gonad enables them to be marketed all year, something that cannot be done with diploids (Allen, 1988). Although triploid and diploid oysters exhibit similar Scope for Growth (SFG), they may partition this SFG energy in different proportions to different body tissues (Kesarcodi-Watson et al., 2001). Gametogenesis in bivalves usually involves mobilisation of reserves from the adductor muscle to the gonad, but in high-food environments such transfer may not be required. In such situations the adductor muscle weight and biochemical content may be similar between triploids and diploids. Racotta et al. (2008) demonstrated this situation in the lion-paw scallop, *Nodipecten subnodosus*, but also identified the possible transfer of certain highly unsaturated fatty acids (*e.g.* 20:4n-6 and 20:5n-3) from the adductor muscle to the gonads in both triploids and diploids. Brake et al. (2004) found significantly increased shell length of triploid compared with diploid common mussel (*Mytilus edulis*) after 9 months in a high-growth environment, but only after 23 months in a low-growth environment. They also reported that almost all triploids were male adding to growing evidence for highly skewed sex ratios in triploid bivalves (Brake et al., 2004).

Harding (2007) compared growth rates of triploid and diploid eastern oysters from a disease-resistant strain (DEBY) with growth rates of triploid Suminoe oysters. Triploids from both species had higher growth rates than the diploid eastern oysters with triploid Suminoe oyster reaching market size (76 mm) in 1.1 yr, triploid DEBY in 1.2 yr and

diploid DEBY in 1.5 yr. In Paynter et al.'s (2008) field comparison between triploid American oyster and triploid Suminoe oyster, the non-native oysters showed significantly faster growth than the native oysters, but it should be noted that this is not a comparison between triploids and diploids within a single species.

For shellfish other than bivalves, Zhang et al. (1998) determined that the growth of triploid juvenile Pacific abalone up to 4 months was significantly greater than in diploids, while Sun et al. (1992) had only seen higher triploid growth in the second year (Table 3). The specific growth rate of juvenile triploid Chinese shrimp, *Fenneropenaeus chinensis*, was shown to be lower than that of diploids in a full sib family (Zhang et al., 2008), irrespective of salinity stress. However, when mature this species usually showed increased growth of triploids (Xiang et al., 2006).

6.4. Deformities

The incidence of deformities is an important topic regarding the prospects for farming triploids as well as from an animal welfare perspective and, last but not least, for public acceptance of market triploids. Zanuy et al. (1994) described a high incidence of deformities in triploid European sea bass larvae that died just after hatching, and Sugama et al. (1992) considered that high numbers of deformities in triploid red sea bream contributed to their lower survival. Sutterlin and Collier (1991), McGeachy et al. (1996) and Sadler et al., (2000c) considered lower jaw deformities in triploid Atlantic salmon due to the triploid condition itself rather than to the induction shock applied to eggs. However, Sadler et al. (2000c) also noted that triploids were more affected than diploids by skeletal, opercular and gill filament malformations and presented a reduced gill surface area but

1096 they found impossible to separate both effects from triploidy itself and shock damage. Other studies showed an overall low incidence of external vertebral axis deformities
1098 (Oppedal et al., 2002; Cotter et al., 2002), although higher in triploids than in diploids early in development but lower in triploids at slaughtering. Triploid rainbow trout embryos
1100 and larvae have a higher incidence of deformities (macrocephalia, lordosis and twisted body) than diploids (*e.g.*, Solar et al., 1984; Myers and Hershberger, 1991). Further,
1102 Madsen et al. (2000) observed a 15% to 20% incidence of spinal deformities in triploid fish (no data for diploid controls). A higher rate of eye cataracts in triploids was recorded
1104 by Cotter et al. (2002) and by Oppedal et al. (2002) in Atlantic salmon. Eye cataracts did not significantly affect production characteristics (Cotter et al., 2002).

1106

The number of deformities observed in triploids seems to be related to the method used to
1108 produce them. Triploid trout produced by crossing a tetraploid male with a diploid female have similar abnormal embryo rates to diploid controls (Myers and Hershberger, 1991),
1110 suggesting that at least some of the abnormalities detected in triploids in other studies might be due to the treatment rather than to triploidy itself. In rainbow trout, temperature
1112 shocks induced higher mortality at the eyed or hatching stages, and a higher rate of deformities at hatching than pressure shocks, which showed no significant difference in
1114 survival, growth or deformity to diploids throughout the hatchery stages (Haffray et al., 2007). Further, maternal effects on the type of malformation at this stage, potentially
1116 associated with egg quality, were also observed.

1118 Triploidisation may also affect the development of vertebrae (Deschamps et al., 2008). Triploid rainbow trout had one extra vertebra with an otherwise normal mineralization rate
1120 and bone density (Kacem et al., 2003). In Indian catfish, *Heteropneustes fossilis*, the

number of vertebrae was significantly reduced and the total surface area of the air sac was
1122 1.6 times as large in triploids compared with diploids (Tiwary and Ray, 2004). On the other hand, triploid European sea bass had a similar incidence of vertebral deformities to
1124 diploids but the severity of these deformities was higher (J.-C. Falguière, unpublished data).
1126

In conclusion, the majority of studies suggest that the physical or chemical manipulations
1128 are the main cause of the higher incidence or severity of deformities and the lower larval survival observed in triploids. Both variables are presumably inversely correlated, since
1130 more deformities likely result in lower survival. However, some studies suggest that the triploid condition is the main cause of deformities. Thus, depending on the type of
1132 deformities considered, triploidy could increase their frequency during early development.

1134 *6.5. Behaviour*

1136 Altered behaviour is frequent in triploid fish. Unusual swimming and feeding behaviour was observed in triploid rainbow trout larvae (Myers and Hershberger, 1991; Solar et al.,
1138 1984). In Atlantic salmon, McGeachy et al. (1995) observed triploid larvae in a state of prostration, while Carter et al. (1994) showed that triploid parr exhibited more severe fin
1140 damage than diploids, indicative of abnormal swimming behaviour. Fin erosion is of concern because its potential impact on survival and welfare perception (Huntingford et
1142 al., 2006). Agonistic behaviour assessed by video observation of number of chases and counterattacks was no different between triploids and diploids in three strains of rainbow
1144 trout (Wagner et al., 2006). Triploid fighting fish, *Betta splendens*, showed less aggressive behaviour (erection of fins or opercula, air gulping, undulating movements, striking and

biting) than diploids, presumably due to their sterility (Kavumpurath and Pandian, 1992). Groups of diploid, triploid and diploid mixed with triploid chinook salmon, *Oncorhynchus tshawytscha*, were compared for their performance. Triploids were found to be less aggressive during feeding than diploids or mixed fish, and although there was no difference in growth among the three groups, cortisol levels in diploids were lower when compared to those of fish from the other two groups (Garner et al., 2008). Because little is known about the agonistic competence of triploids in contact with diploids or between triploids, such characteristics should be investigated in other commercial species.

6.6. Reproduction

6.6.1. Fish

In autotriploid fish, meiosis is seriously affected because three homologous chromosomes cannot correctly pair during the zygotene stage of prophase I (Carrasco et al., 1998; Cuñado et al., 2002). This impairment interferes with gonadal development and gametogenesis in almost all aquacultured species examined so far, but differentially between sexes. The reproductive performance of triploids for the main cultured species of fishes, shellfish and crustaceans is summarized in Table 3.

In females, entry into meiosis marks the transition from oogonia to oocytes, which precedes follicular assemblage and oocyte growth by yolk deposition (Benfey, 1999). Hence, ovaries of triploid females remain highly reduced in size and weight. This results in a lower gonadosomatic index (GSI) and may also imply a higher dressing percentage (carcass yield at slaughtering) due to the diversion of energy from vitellogenesis to body

growth. As observed above, this excess energy result in that ovaries may be hidden in deposits of perivisceral fat in the abdominal cavity (Flajšhans, 1997). This partially counterbalances the advantage issued from lowering GSI in terms of carcass yield. Thus, the lower GSI of triploids does not always mean a higher dressing percentage if there is excess visceral fat. Macroscopically, ovaries of triploids appear paler and more transparent than those of diploids owing to the absence of yolk and increased proportions of connective tissues (Fig. 5). Microscopically, they contain only small numbers of oogonia and very few developing primary oocytes (Hussain et al., 1996; Benfey, 1999; Felip et al., 2001c; Devlin and Nagahama, 2002). Thus, triploid females rarely produce eggs but, if they do, eggs are usually very few, undeveloped and unfertilisable (Benfey and Sutterlin, 1984b; Piferrer et al., 1994a; Penman et al., 1987; Brämick et al., 1995; Gillet et al., 2001). The typical gonadal histology of triploid females as compared to that of diploids, as it appears in the European sea bass, is illustrated in Fig. 6. It has been suggested that failure of oocyte growth may also reflect genomic imbalances due to the presence of an extra set of chromosomes (Krisfalusi et al., 2000). In rainbow trout, few studies on sex differentiation and sex reversal showed that the genetic sex of the gonad did not appear to influence the degree of sterility, since hormonally inverted XXY fish with ovaries and XXX fish with testes showed similar characteristics to their non-inverted counterparts (Lincoln and Scott, 1983; Krisfalusi and Cloud, 1999; Devlin and Nagahama, 2002).

Although triploid female fish do not produce mature oocytes around the time of first sexual maturation of diploids some studies report the occasional production of mature oocytes in older triploids (Benfey, 1999). For example, Manning et al. (2004) found some vitellogenic oocytes in 40-month-old triploid yellowtail flounder, *Limanda ferruginea*,

females and evidence of prior summer ovulatory activity. These oocytes were probably aneuploid and therefore non-viable.

In contrast, in males meiosis takes place with the onset of puberty after spermatogonia have gone through many rounds of cell division by mitosis. Thus, although meiosis is also impaired, the testes of triploid males can develop up to a size similar to those of diploids (Fig. 5), with a considerable population of fully functional steroidogenic cells. Hence, their dressing percentage may be similar to that of diploids. Histologically, spermatogenesis in triploids may exhibit spermatogonia multiplication and spermatocyte divisions, but infertility is expected to result from random segregation of trivalents followed by (potential) production of aneuploid sperm (Benfey et al., 1986; Ueda et al., 1987; Hussain et al., 1996; Benfey, 1999; Felip et al., 2001c; Devlin and Nagahama, 2002; Haffray et al., 2005). To illustrate the typical gonadal histology of triploid and diploid males, the situation in the European sea bass is depicted (Fig. 7).

Functional sterility, *i.e.*, no sperm production, has been confirmed in the autotriploid males of several aquaculture species, including the European sea bass (Felip et al., 2001c; Peruzzi et al., 2004), turbot (Cal et al., 2006), barfin flounder, *Verasper moseri* (Mori et al., 2006), gilthead sea bream (Haffray et al., 2005), and Arctic charr (Gillet et al., 2001). On the other hand, autotriploids of some species are capable of producing small amounts of spermatozoa, but they are aneuploid and thus incapable of generating viable offspring if used for fertilisation. These include the Atlantic salmon (Benfey and Sutterlin, 1984b), rainbow trout (Benfey et al., 1986), coho salmon (Piferrer et al., 1994a), Thai silver barb, *Puntius gonionotus* (Koedprang and Na-Nakorn, 2000), and tench (Linhart et al., 2006).

An interesting issue related to the possible genetic impact of triploid males (section 8) is related to the physiology of their sperm. In this regard, sperm produced by triploid tench contained lower initial frequency of motile spermatozoa when compared to the sperm produced by diploids, but their frequencies began to equilibrate after 60 s (Linhart et al., 2006). In contrast, their initial similar velocities began to differ after 45 s in favour of spermatozoa of triploids (Fig. 8). In a few cases, spermatozoa from autotriploid males could carry out egg activation leading to non-viable aneuploid embryos, as in rainbow trout (Lincoln and Scott, 1984); plaice, *Pleuronectes platessa* (Lincoln, 1981a); common carp (Cherfas et al., 1994) and fighting fish (Kavumpurath and Pandian, 1992). For Atlantic salmon, there are no reports of eggs being fertilised by sperm from triploids. Thus, the physiology of sperm in those species able to produce even after triploidy induction deserves further attention. In summary, induced triploidy in general confers genetic sterility. However, in some rare cases viable larvae were obtained after insemination of normal or aneuploid eggs from a triploid female with sperm from triploid males, as in grass carp (Goudie, 1988; van Eenennaam et al., 1990), yellowtail flounder (Manning et al., 2004), and tench (Linhart et al., 2006).

Since the testes of triploid males have functional steroidogenic cells, they experience the hormonal changes and, thus, the negative effects associated with sexual maturation observed in diploids. Therefore, to produce completely sterile fish with greater carcass value that do not produce secondary sexual characteristics, it is necessary to combine the induction of triploidy with endocrine feminisation (Piferrer, 2001). This can be achieved by the hormonal feminization of triploids (Piferrer et al.,1994b) or, with more certainty, by the triploidisation of all-female stocks (Lincoln and Scott, 1983). This, along with

consideration related to market size, is why most farmed trout stocks are female and triploid (section 7).

In Thai walking catfish, *Clarias macrocephalus* x African catfish crosses, gametes were produced by both diploid and triploid hybrids, though with different generative potentials: sperm from both diploid and triploid hybrid males fertilised eggs of the maternal species, but no fry survived to the yolk absorption stage, whereas eggs of both diploid and triploid hybrid females were fertilised with sperm of the paternal species, but only the diploid progeny survived after yolk absorption stage (Na-Nakorn et al., 2004). In mud loach x cyprinid loach crosses, diploid hybrids of both sexes reached sexual maturity, while triploid hybrids of both sexes were found to be sterile with abnormal and significantly reduced gonadal development (Park et al., 2006).

6.6.2. Shellfish

In shellfish, triploidy does not necessarily produce complete sterility, but rather a decrease of gonadal development. Functional gametes and sometimes spawning have been recorded in triploid shellfish, although at very low rate, such as in Japanese pearl oyster, *Pinctada fucata martensii* (Komaru and Wada, 1990); dwarf surfclam (Guo and Allen, 1994b); Pacific oyster (Allen and Downing, 1986); American oyster (Allen, 1987); Manila clam, *Tapes philippinarum* (Shpigel and Spencer, 1996); Suminoe oyster (Erskine and Allen, 2003); and Pacific abalone, *Haliotis discus hannai* (Li et al., 2004). In some species, such as the Japanese pearl oyster (Komaru and Wada, 1990) or in the Pacific oyster (Guo and Allen, 1994c), eggs from triploids were found to be fertilisable and to proceed through development (Komaru and Wada, 1990; Guo and Allen, 1994c; Utting et al., 1996).

Fecundity in triploid Pacific oysters is estimated to be 2% that of diploids (Guo and Allen, 1994c) and production of eggs from triploid Manila clam was reduced to 12.5% of that of diploids (Utting et al., 1996).

On the other hand, triploid bay scallop, *Argopecten irradians*, showed reduced gonadogenesis and failed to ripen during the spawning season of the species (Tabarini, 1984). Different degrees of gametogenesis, but no formation of ripe eggs or spermatozoa were reported in scallop, *Chlamys nobilis* (Komaru and Wada, 1989); catarina scallop, *Argopecten ventricosus* (Ruiz-Verdugo et al., 2000); common mussel (Brake et al., 2004); Mediterranean mussel, *Mytilus galloprovincialis* (Davis, 1997); soft shell clam, *Mya arenaria* (Allen et al., 1986); Quahog, *Mercenaria mercenaria* (Eversole et al., 1996); and Sydney rock oyster, *Saccostrea commercialis* (Cox et al., 1996). In summary, in some triploid bivalves functional gametes may be produced, but at a low rate thus limiting, but not preventing their spawning capacity. Variation in results from different studies on bivalves may be the consequence of differences in food availability (Racotta et al., 2008).

6.7. Processing yield and flesh quality

Sexual maturation affects flesh quality in many species by diverting energy (lipids in fish or glycogen in molluscs) into reproduction. Sterilisation by triploidisation affects differentially body morphology, processing yields (gutting, filleting, trimming) and flesh quality (lipids or glycogen, flesh colour, taste, texture).

Many induced salmonid triploids generally show a lower condition factor than diploids at commercial weight (*e.g.*, Galbreath and Thorgaard, 1995; Withler et al., 1998; Bonnet et al., 1999; Gillet et al., 2001) as do triploid gilthead sea bream (Haffray et al., 2005),

European sea bass (Felip et al., 2001b; Peruzzi et al., 2004) and shi drum (Segato et al., 2007). In the European sea bass, for example, the condition factor is lower because triploids are comparatively smaller in weight than in size when compared to diploids.

Triploid females have generally a better dress-out percentage (a.k.a. carcass yield) during the reproductive season due to their lower gonadal development, as observed in rainbow trout (Quillet et al., 1986), common carp (Basavaraju et al., 2002), Arctic charr (Gillet et al., 2001), European sea bass (Peruzzi et al., 2004), and gilthead sea bream (Haffray et al., 2005). However, depending on the species, size and age at maturity, triploids may have a similar dress-out percentage as diploids (Sheehan et al., 1999), although this can be negated by a higher accumulation of visceral fat (Quillet et al., 1986). A ploidy x sex interaction in gutted yield is also often noticed in several species, mainly during the reproductive season, as triploidy affects the two sexes differentially (Peruzzi et al., 2004). Nevertheless, gender differences in gutted yields were neither seen in common carp (Basavaraju et al., 2002) nor in Arctic charr (Gillet et al., 2001). Regarding the yield of fillets, this can be higher in triploids than in diploids (European sea bass: Peruzzi et al., 2004; rainbow trout: Werner et al., 2008), or similar to diploids (gilthead sea bream: Haffray et al.,2005).

Analysis of muscle growth and development in triploid Atlantic salmon showed lower density of satellite cells, reduced rates of fibre recruitment, hypertrophy of muscle fibres, advanced development of myotubules, myofibrils and acetylcholinesterase staining at the myosepta compared with diploids (Johnston et al., 1999) but no differences in gaping intensity, fillet texture, post-mortem end pH or crude chemical composition (Bjornevik et al., 2004). Triploid rainbow trout also had reduced muscle cell number (fibre hypertrophy)

(Poontawee et al., 2007). No differences related to ploidy level were observed in composition or flesh colour in shi drum when fish were compared at the same body weight (Segato et al., 2006). Differences in fatty acid composition were observed between diploid and triploid tench (Buchtova, 2004), but the comparison was not performed at the same body weight. Triploids may have a lower fat content in the fillet (Peruzzi et al., 2004; Haffray et al., 2005; Werner et al., 2008) outside the reproductive season, but once reproduction starts the flesh of triploids does not suffer of fat decrease as that of diploids do (Quillet et al., 1986).

Pan-sized triploid rainbow trout have a capability to fix canthaxanthin in the flesh that is similar to diploids (Choubert and Blanc, 1985; Choubert et al., 1997). However, once reproduction starts, canthaxanthin fixation efficiency becomes reduced in diploids (Choubert and Blanc, 1989), conferring an advantage to triploids for processors and consumers.

6.8. Improvement of triploids by genetic selection

As triploids are generally sterile, they cannot be directly improved by selection through successive generations. However, they could theoretically be improved by the selection of their diploid parental lines if traits measured in diploids are the same as in triploids. Only few studies have investigated genetic parameters in polyploid fish and genetic correlations with diploids. This research requires the production of families to estimate the variance component due to either environmental or genetic sources and their possible interaction. When comparing diploid and triploid families, the “environmental” source of variation includes non-genetic maternal effect (egg quality, egg size) and also some other sources of variation, such as tank effects. The genetic sources of variation can be associated with the

presence of the extra maternal set of chromosomes and can involve simple gene dosage (additivity) between chromosome sets or positive or negative dosage compensation effects (heterosis), epigenetic mechanisms, and transcriptional co-suppression (negative gene dosage compensation). Studies on gene dosage compensation in the allotriploid endemic Iberian minnow showed that the allelic expression patterns differ between genes and between different tissues (Pala et al., 2008). Thus, it appears that in triploids rather than a whole haploid chromosome set (haplome) being silenced, regulatory mechanisms involve selective individual gene-copy silencing.

Several approaches have been used to compare the performance of diploid and triploid full-sib families (Choubert and Blanc, 1985; Withler et al., 1995; Withler et al., 1998; Friars et al., 2001; Oppedal et al., 2002; Johnson et al., 2004; Shrimpton et al., 2007). Generally, these experiments identified significant family by ploidy ("G x T(riploidy)") interactions for traits such as growth, condition factor, sensitivity to artificial photoperiods, etc. However, with this approach family effect includes genetic effects and non-genetic maternal effects, since only full-sibs were tested. Moreover, they were conducted on a very limited number of families (n = 4–12), limiting the range of genetic variability investigated. A second type of approach attempted to elucidate the relationship between the two ploidy types by adapting mating design to more precisely evaluate the additive genetic component. The production of paternal half-sib families obtained by mixing eggs from different dams before fertilisation (in order to avoid non-genetic maternal effects) and subsequent fertilisation of sub-groups of eggs by different sires allowed estimation of additive differences between sires (n = 12 –31). Using this approach, Choubert et al. (1997), Bonnet et al. (1999), Blanc and Vallée (1999), Blanc et al. (2001; 2005) observed only very limited G x T interaction for growth, survival or flesh

colour in rainbow trout and brown trout. They concluded that the small amount of G x T interaction observed does not justify adoption of specific breeding programs to improve triploids. Blanc et al. (2005) noted that each haploid chromosome set could make a separate additive genetic contribution to growth in triploids (defined as the mean of the sum of the breeding values of the three chromosome sets divided by the number of chromosome sets of the progeny) and that therefore the selection of parents of triploids could be important. Recently, Johnson et al. (2007) by using 62 half-sib families of chinook salmon calculated the first estimates of heritability based on the breeding value of the parents for growth and survival, showing that they do not differ between diploids and triploids, thus confirming the Blanc et al. (2005) observation. Finally, Bonnet et al., (2002) did not observe G x T interaction on growth or several quality traits (body morphology, dressing and fillet yields, fat content in the muscle) in brown trout, and concluded that selection for growth in diploids is not likely to generate major different responses for the above quality traits.

These preliminary studies are restricted in scope, but improved protocols involving more families and pedigree information will enable better estimations of the genetic parameters and genetic correlation between ploidy level, in order to establish whether it is appropriate to select diploid lines for the performance of triploid progenies.

7. Application of triploidisation in aquaculture

Polyploids have many useful applications to aquaculture. It should be noted that, according to national and EU regulations (Directive 90/220/CEE of 23 April 1990), polyploids, like hybrids, are not considered to be genetically modified organisms (GMOs).

Thus, polyploids are exempt from the stringent regulations applying to the use and containment of GMOs in farming.

7.1. Fifty years of research and development of triploidy in fish

Although domestication of fishes started centuries ago (Balon, 2004), triploidisation was first investigated in fish in the stickleback, *Gasterosteus aculeatus* (Swarup, 1959), and research on polyploidisation in aquaculture species began in the 1970–80's in the United Kingdom (UK), USA and Canada. Then it was followed by work in salmonids, mainly in rainbow trout in France, USA and the UK, and in Atlantic salmon in the UK and Norway, and in molluscs in France and the USA, where the method to produce tetraploid oysters is currently patented. In the late 1990s, similar techniques began to be applied to marine fish, like European sea bass, gilthead sea bream, turbot, and halibut, *Hippoglossus hippoglossus*, in Spain, France, Italy, Israel and Canada.

The fish species in which triploidy is commercially used include the rainbow trout in USA, Canada, France, Japan, UK, Korea, Iran, Turkey, Poland and Chile; brown trout in UK and France; brook trout in Canada and France; Atlantic salmon in Canada and Chile; Arctic charr in France, Canada, Iceland and Austria; chinook salmon in Canada; amago salmon, *Oncorhynchus rhodurus*, masu salmon, *O. masou*, coho salmon, ayu, hirame, *Paralichthys olivaceus*, and cyprinid loach in Japan; and grass carp in the USA (Arai, 2001; Hulata, 2001; Rothbard, 2006). The main commercial shellfish species that is triploidised is the Pacific oyster, but triploid scallops, clams and mussels have also been produced at least on a semi-commercial scale (Beaumont and Fairbrother, 1991).

The aquaculture production of allotriploids includes the market-preferred spotless allotriploid rainbow trout x amago salmon, *Oncorhynchus rhodurus*, hybrids (Hattori and Seko, 1999), sterile allotriploid Thai walking catfish x African catfish hybrids (Na-Nakorn et al., 2004), allotriploid chum salmon, *Oncorhynchus keta* x whitespotted charr, *Salvelinus leucomaenis*, hybrid with improved survival or rainbow trout x masu salmon, *Oncorhynchus masou ishikawa,* and rainbow trout x whitespotted charr allotriploid hybrids with improved survival, growth and with less developmental abnormalities than their diploid counterparts (Arai, 2001).

In addition to the primary goal of increased growth by diverting energy from gonadal maturation, triploids have other applications (Colombo et al., 1997). Allotriploids not only may exhibit higher survival, hybrid vigour and sterility than their corresponding diploid hybrids, as discussed above, but also greater resistance to some viral diseases (Parsons et al., 1986; Dorson et al., 1991). A new application of triploidy is concerned with the xenotransplantation of the germ line. Thus, triploid salmon xenotransplanted with rainbow trout primordial germ cells was shown to produce trout offspring (Okutsu et al., 2007). Xenotransplantation of the germ line could facilitate the aquaculture of species such as bluefin tuna, *Thunnus thynnus,* which because of their size present important challenges for broodstock management. In this new application, triploidy would be used to prevent the surrogate species producing its own gametes.

7.2. Application of triploidisation in EU aquaculture

Application of triploidisation in aquaculture in Europe is currently limited to the production of table fish (salmonids) and shellfish (Pacific oyster). Triploidy is also applied to salmonid restocking for fishing in freshwater.

7.2.1. All-female stocks in trout production

The main application of triploidy in European fish culture is in rainbow trout farming. Trout production is highly diversified in relation to rearing conditions (freshwater, seawater or brackish water), containment facilities (ponds, raceways, tanks or floating cages) and in size at slaughtering (from 350 g to 1.2 – 3 kg).

In freshwater, rainbow trout grows fast in its first year of life, but may encounter early sexual maturation of males at one year old, with consequent deterioration of meat quality (reduced contents of protein and lipids, poor flesh pigmentation) and increased mortality. Since the 1980s, this problem has been solved for the pan-size, whole product (initially 250 g, nowadays 350 g) by the use of all-female XX diploids. All-female stocks are produced by crossing normal females with genetic females sexually inverted with androgens (Piferrer, 2001). This treatment is performed according to the EU Directive 96/22/CE (29 April 1996) under the responsibility of a veterinarian and upon farm declaration to the national authority. Treated fish need to be tagged and are not allowed to be sold for consumption. Today, nearly 80% of the European trout production in freshwater is based on all-female diploid stocks, even though females grow nearly 10% slower than diploid males up to pan size.

7.2.2. Application of triploidisation in trout production

Overproduction in the 1990's and changing consumer demands for fillet instead of whole product pushed farmers towards the fresh fillet (1.2 kg fish) and smoked fillet (2.5–3 kg fish) markets. However, associated with all-year-round egg production, female sexual maturation began to occur when fish weighed only around 450 g (14–16 months old). This early maturation is highly damaging for fillet production, because it compromises body growth. Thus, to avoid economic risks and to secure their activity, some farmers decided to raise triploid all-female trout, even though triploids (both sexes considered) grow 10–15% less than normal diploids once in production and are more demanding in terms of water quality, low stocking density and good oxygen levels.

Triploidy is induced by either thermal shock (at 26°C) or pressure treatment (65 MPa) using specifically designed 3–10-liter pressure chambers in order to block the extrusion of the second polar body after fertilisation. Triploidisation success can be assessed by flow cytometry at the eyed-egg or hatched-fry stages, or by a fin sample (Allen, 1983; Lecommandeur et al., 1994). In France, the triploid percentage gradually improved from 89% (n = 12 production batches) in 1997 to 98% in 2006 (n = 59). Although most treatments are nearly 100% efficient, routine ploidy tests are used to enable the identification of occasional errors, such as the inadvertent mixing of diploid and triploid eggs after grading at the eyed stage (Haffray et al., 2004).

Presently, the total European triploid trout production is estimated at 15,000 tons. Fillets of high quality are marketed all year round, especially during wintertime, when the demand for smoked products is at its height. Since at this time of the year any mature trout would be an economic loss for the processor, the inferior growth of triploids is largely

compensated by their sterility. Thus, triploid trout culture, initially promoted by farmers, is now promoted by processors. The superior quality of triploid trout fillet was recognized in 2007 as a condition to obtain the “Label Rouge”, the highest official recognition of quality for the smoked trout fillet in France.

Besides rainbow trout, triploids of other salmonids, such as brown trout, brook trout and its hybrids, and Arctic charr, are also used for restocking 250–2500 g fish by angling associations in different countries (France, UK, Germany and Austria).

7.2.3. Application of triploidisation in Pacific oyster production

Pacific oyster production in France has been based on the collection of wild spat since the naturalisation of this introduced species in the 1960s and 1970s. Its main market is the winter market near Christmas time (60%) and has benefited from the introduction of triploids since late 1996. Initially directly induced by cytochalasin-B treatment (Beaumont and Fairbrother, 1991), most of the European production of triploid oyster relies now on the cross between tetraploid males and diploid females, according to the patented Guo and Allen (1994a) method, applicable to all bivalve species. The efficiency of this method is 100% and triploid oysters thus produced have two sets of chromosomes from the father and one set from the mother. In Europe, nearly 20% of the production is now based on triploid oysters, while in the USA around 50% of cultured Pacific oysters are triploids. These are virtually sterile and the risk of their potential reproduction in the sea with triploids or diploids is estimated to be very low, given their poor reproductive performances. In France, tetraploid lines are being produced and held in quarantine by IFREMER under strict environmental constraints and controls by the national authority, as

told above. Diploid semen is being sold to the hatcheries to fertilize the eggs from their diploid maternal broodstocks. Triploid oysters grow faster, can be sold year round and provide a firmer, more palatable product compared with diploids (Allen and Downing, 1991; Nell, 2002).

7.3. Society perception about the induction of triploidy in aquaculture species

The application of triploidy in rainbow trout and Pacific oyster farming illustrates the society's perception of triploidy in aquaculture (Komen et al., 2002). The implementation of triploidy in trout and oyster farming was mainly producer- and processor-driven in response to consumer demands for highest quality or ready-to-cook products. Preliminary trials growing triploids of European sea bass, gilthead sea bream and turbot were performed in the late 1990s by some farmers, but there is limited information on the true benefits of triploids under normal farming conditions for many species. For example, there is no study on performance or behaviour of triploid European sea bass or gilthead sea bream in commercial cage culture, a method that has high escape risk. Although stocking density and levels of dissolved oxygen can be critical factors in the farming of oyster and trout triploids, further research is needed into, for example, malformations (in salmonids), reversion to diploidy (in oysters, Allen et al., 1999; Chandler et al., 1999; Calvo et al. 1999) and disease resistance (in both salmonids and oysters)

The French Food Safety Authority, AFSSA, has been requested by the French Competition and Fraud Authority (DGCCRF) and by a consumer association to investigate the differences between the diploid and triploid oysters in order to respond to consumer concerns. AFSSA reports that triploid oysters have been consumed for many years without

problems (declaration of 23/11/2001) but has recommended further study into the uptake and retention of pollutants, heavy metals, algal toxins or pathogenic bacteria by triploid relative to diploid oysters (AFSSA, 2001).

The SEFABAR (Sustainable European Farm Animal Breeding and Reproduction; www.sefabar.org) research project on the perception of animal breeding by the European society revealed the existence of contrasting views concerning the application of breeding and reproductive technologies to farm animals. Clear presentations of the facts about the costs and benefits of these technologies are required to stimulate the debate among citizens, and this debate must be supported by advice and recommendations from experts in independent governmental agencies. Animal welfare organizations also have a significant role to play (Kolar and Rusche, 2003) since, depending on country, species or farming systems, socio-economic benefits of farming triploids need to be evaluated and society perception evaluated. Thus, in the application of triploidy to aquaculture, expected advantages should be carefully balanced by predicted disadvantages (Table 5).

8. Performance of triploid fish in the natural environment

The reproductive interactions between farmed escapees and wild conspecifics have been studied in diploids of different fish species. Emphasis has been placed in three different aspects: the ecological consequences of escape, the sexual behaviour of farmed fishes in the wild, and the effects of their genetic introgression within wild populations (Hindar et al., 1991a,b; Youngson et al., 2001).

The value of triploid fish to reduce or avoid genetic interactions between cultured and wild stocks requires an evaluation of their behaviour and performance in the natural environment. However, little information is available on this topic (Table 6).

An early study by Solar et al. (1986) demonstrated that hormonally-sterilised diploid coho salmon released into the wild showed a lack of homing behaviour when in the marine environment, but still could display sexual behaviour and thus could interfere with the reproduction of native spawners.

Common garden experiments involving tagged fish released into the wild can be a promising approach to investigate triploid-diploid interactions under a free-ranging situation, but at present this information is not yet available for most aquacultured species.

Experiments on the intentional release of triploid fish are restricted to Atlantic salmon and rainbow trout. Ocean migration studies on salmon in Ireland revealed that male triploids returned to their natal area in nearly the same proportions as diploids, whereas triploid females mostly did not. The few females that migrated did so passively entrained with diploids, thus reducing potential threats to wild salmon in those areas (Wilkins et al., 2001). Similar results were found in another trial in which the return rate of triploid salmon to the coast and in freshwater was substantially reduced, indicating that, owing to their low reproductive ability, the chances of interbreeding with wild stocks were very low (Cotter et al., 2000). With steelhead rainbow trout, on- and off-rearing site releases of triploid fish resulted in homing behaviour, suggesting site fidelity with seasonal effects (Bridger et al., 2001).

Less attention has been paid to the use of triploids as a genetic conservation measure in non-anadromous salmonids. In the USA, Dillon et al. (2000) did not find differences in fishing success between mixed-sex diploid or triploid rainbow trout in 18 Idaho streams. Teuscher et al. (2003) investigated also the growth and the survival of triploids until 51 months of age in two Idaho water reservoirs (150 ha, water depth < 15 m). They reported a growth similar to that of diploids and 38–94 % better survival of triploids at the end of the experiment, which they attributed to their lower mortality due to lack of maturation. Wagner et al. (2006) also did not observe differences in survival between triploids and diploids from three hatchery stocks after wintering in small ponds. However, if sterile rainbow trout can be stocked as catchables in streams, their release in high mountain lakes may require adjustments or may eventually prove problematic because of lower survival rates, indicating that the use of triploids for recreational fisheries needs further investigations, considering also the ecological interactions (Kozfkay et al., 2006).

The potential use of triploid brown trout was investigated from 2003 to 2006 by the UK Environmental Agency to evaluate its contribution to the good ecological management of freshwater bodies forecast for 2015, as defined in the EU Directive 2000/60/CE of 23/10/2000 (http://www.environment-agency.gov.uk/business/sectors/39903.aspx). Three steps were followed: bibliographic survey, risk analysis of restocking with triploids and common garden experiments. These were carried out in lakes, rivers and ponds, and compared the survival, growth, catchability, feeding regimes and reproductive behaviour of all-female triploids with respect to wild or domesticated diploids. Results showed that in general all-female domesticated triploids 1) had a similar catchability than all-female domesticated diploids, 2) their restocking in rivers and ponds did not noticeably impact wild populations, 3) had the same feeding regime than domesticated and wild diploids, 4)

did not exhibit reproductive migration to spawning grounds, and 5) had a higher rate of capture at the end of the commercial fishing season. After a public consultation, it was concluded that triploids could be used for recreational fisheries. The implementation plan fixed as an objective to reduce the use of fertile diploids —by 30% in 2010 and by –50% in 2013— until their replacement with triploids by 2015.

In general, triploids grow slower than diploids when reared together, especially in sub-optimal conditions, suggesting that they are at a disadvantage in comparison with wild diploids in case of escapement into natural waters. Nevertheless, the assessment of their actual performance in the wild in terms of trophic competition and reproductive interference by male triploids is only beginning to be investigated and remains an unexplored quest and a challenging task for future research. To summarize, then, the release of triploid fish in open ecosystems will probably be affected at least by the same lower viability than domesticated diploids. This can be associated with the benefit of significant reductions of interbreeding with native stocks. Thus, triploidy, possibly combined with feminisation, can significantly diminish the ecological and genetic impacts of farmed fish on wild populations.

9. The potential use of induced triploidy for the reproductive, genetic and transgenic containment of cultured fish

In fish and shellfish culture, sterility associated with triploidy may be exploited to provide containment of domestic stocks. However, direct triploidy induction is seldom 100% consistently effective and this creates a problem in relation to genetic containment. In the

1642 case of oysters, even with 100% triploids deployed, reversion to diploidy is an inherent problem (Allen et al., 1999; Chandler et al., 1999; Calvo et al. 1999).

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Farming of sterile animals in aquaculture is advocated by several non-governmental 1646 organisations (NGOs) and national or international independent agencies to limit genetic interactions in two contexts: 1) from escapees, as indicated by the North Atlantic Salmon 1648 Conservation Organisation (NASCO; Resolution of June 1994), the Food and Agriculture Organisation (FAO; Technical Guide for Responsible Aquaculture, art. 9.3, pp. 21–22), 1650 and the International Council for the Exploration of the Seas (ICES; Code of Good Practices for the Introduction and Transfer of Marine Organisms, 1994); see also Hansen 1652 et al. (2007); and 2) from deliberately introduced fish into freshwater systems for fishing as indicated by the UK Environmental Agency since 2003, and the Conseil Supérieur de la 1654 Pêche (France) since 1994. A recent survey across the USA (Kozfkay et al., 2006) showed that at least ten states have ongoing programs for sterilising hatchery salmonids to 1656 preserve native species. The use of sterile triploids has also been proposed as a solution to the problem of the containment of GMOs (Donaldson et al., 1993; Youngson et al., 2001; 1658 Rasmussen and Morrissey, 2007).

1660 *9.1. Triploidy and reproductive containment*

1662 Reproductive containment may be required to constrain the excessive multiplication of highly prolific species, that otherwise would overcrowd ponds with small stunted fish. 1664 This is the case of Nile tilapia but the dependence of production upon natural spawning has made the use of triploids less convenient than all-male culture by androgen sex-1666 reversal or YY-male-technology (cf. Toguyeni et al., 2002; Tariq Ezaz et al., 2004).

Reproductive containment would also be a safeguard against the threats of competition or predation imposed on native populations by escapees of exotic species introduced under relaxed regulations for aquaculture purposes. In the past, the drive towards profitable aquaculture has generally ignored the potential costs of such biodiversity contaminations, which are often aggravated by the concomitant transfer of alien pathogens and parasites (Naylor et al., 2001; Ruesink et al., 2005).

The growing opposition to the unrestricted introduction of non-native species for aquaculture also brings criticism about the applicability of triploidy alone as a method to overcome the survival and prevent propagation of non-native escapees in the natural environment. Due to the possible inclusion of a small number of fertile diploids within triploidised stocks, and in the case of oysters, reversion to diploidy (Allen et al., 1999; Chandler et al., 1999; Calvo et al. 1999), the dissemination of foreign species outside culture facilities cannot be completely prevented. For instance, different species of Asian carp, introduced in the USA as theoretical triploids, have actually escaped and reproduced in the wild (Naylor et al., 2001). Conservation agencies now dictate that vendors must individually check the ploidy of carp by erythrocyte volume measurement with an electronic particle counter before introduction (Lee and Donaldson, 2001).

A less onerous technical upgrading would be the use of ploidy-sensitive skin colour to discriminate diploids. In theory, a single dominant colour allele (like red colour in Nile tilapia strains) counteracted by two recessive alleles in a triploid fish may give rise to a different phenotype with respect to diploids.

Rather than a means to justify further introductions of exogenous species, 100% triploidy could help instead to eradicate already widespread invaders, especially those considered as particularly injurious. Assuming that triploid male fish retain in part fully functional Leydig cells, as often observed in several species (Felip et al., 2001c), thus manifesting courtship and spawning behaviours despite their infertile sperm (Kitamura et al., 1991), then restocking with all-male colour-checked triploids would expose naturalized females to unsuccessful mating. This approach recalls the sort of biological warfare undertaken by deploying irradiated mosquitoes to disinfest an area (Esteva and Yang, 2005). Coupling a properly adjusted restocking load with selective fishing pressure, a target water body could eventually get rid of its unwanted guests, a strategy that may be worth testing. By the same token, the release of male triploids could adversely affect the recruitment of wild conspecifics.

9.2. Triploidy and genetic containment

While reproductive containment merely restricts the proliferation of a species because it is excessive or unwanted, genetic containment is aimed at averting contamination of natural genetic diversity. Alterations in allelic frequencies of encoding genes in a wild fish population caused by occasional mixing with slightly divergent allelic assortments from domestic conspecifics is assumed to be eventually normalized with time by natural selection.

Nevertheless, in intensely cultured species, like Atlantic salmon, that in nature are substructured into genetically-differentiated populations reproducing in separate drainages, massive and continuous releases of farmed escapees can flood the original

inter-population genetic heterogeneity, breaking down locally coadapted gene complexes. Although the use of triploids may be recommended where Atlantic salmon has been introduced as a non-native species, like in West Canada and Chile, their use in native waters to protect endangered local populations seems less cost-effective than good hatchery practices relying on genetically representative broodstocks, according to the policy of the Atlantic Salmon Federation of East Canada (Bourke et al., 1997). This is because, apart from the cost of triploidy induction, triploids raised in pens are affected by higher morbidity and mortality than normal salmon (Benfey, 2001). A similar challenge faces the expanding aquaculture of cod, *Gadus morhua*, in Northern Europe. Cod has high fecundity and the production of fertilised eggs escaping from sea cages to the open ocean is viewed with concern for the possible genetic impact on natural populations. Although triploidy has been induced in cod at a laboratory scale (Peruzzi et al., 2007), further research is needed on the large-scale production of triploid cod and evaluation of its performance in commercial facilities.

Moving from the conflict between natural and selected strains to the phenomena of genetic introgression and intergradation by hybridisation, then gene exchange between wild and farmed fish becomes equivalent to a sort of gene transfer, whether occurring between natural varieties or species. A good example, within the brown trout complex, is the interbreeding between the varieties or morphs *S. trutta fario* and *S. trutta marmoratus*, whose genetic divergence began at the onset of quaternary glaciations. Confined to the river plain of Northern Italy, the genetic identity of the small populations of *marmoratus* are now endangered by programmed restocking with *S. trutta fario* of North-European origin farmed for the benefit of sport fishing (Argenton et al., 1992). To preserve *S. trutta marmoratus*, with its distinctive marble phenotype and bigger size but low fitness for

farming, the proposed policy of limited restocking with all-female triploid *S. trutta fario* appears to reconcile the imperative of conservation with fishermen expectations (Borghesan et al., 2006).

On a magnified scale, the same problem exists in North America, where the cross-boundary transfer of regionally endemic fish by human activities is now considered as great a threat to the genetic integrity of native freshwater fauna as the introduction of non-indigenous species from outside the continent (Perry et al., 2002).

Fish interspecific hybridisation may also occur spontaneously in nature (Argue and Dunham, 1999) and aquaculture makes extensive use of artificially reproduced hybrids worldwide. At least thirty interspecific and intergeneric fast-growing hybrids are currently farmed for human consumption, but only about half of them are completely sterile (Bartley et al., 1997). For the other half, there is a risk that escapees may either intercross, establishing a novel hybrid population, or backcross with the parental species leading to complex introgression of genes. To avoid this, triploidy has been successfully associated with hybridisation to sterilize fertile hybrids (Na-Nakorn et al., 2004), but mass-scale production of allotriploid fish is still mostly experimental. On the other hand, although nuclear DNA introgression between hybridizing species is considered to be extremely rare, genetic introgression between Atlantic salmon (2n = 58) and brown trout (2n = 80) through spontaneous reproduction of allotriploids has been found possible in a river of the Basque Country (Castillo et al., 2007), leading to the production of salmon-like offspring bearing some brown trout genes.

Likewise, of greater significance is the phenomenon of heteroploid mosaics and reversion to diploidy (Allen et al., 1999; Chandler et al., 1999; Calvo et al., 1999). Particularly in shellfish, the lack of certainty about the permanent and irreversible sterile status of triploid non-native oysters (or eventually sterile GMO oysters) deployed in the wild creates real difficulties for the use of this technology for genetic containment.

9.3. Triploidy and transgenic containment

Extreme hostility has arisen against the commercial culture of transgenic fish in the western world, even when transgenic lines bear all-fish constructs derived from the same or closely related species (autotransgenesis). This is particularly true for fast-growing growth hormone (GH)-transgenic fish, though not always for the trade of the so-called glowing-in-the-dark aquarium fish carrying a transgene encoding a fluorescent protein (Wong and Van Eenennaam, 2008). Currently, the main concern about transgenic fish addresses the issue of the ecosystemic compatibility of their commercial farming rather than questions of nutritional safety.

It should be noted that the mandatory requirement of field tests to assess ecological risks posed by fertile transgenics is hardly applicable, either in real ecosystems, wherefrom fish cannot be subsequently eradicated, or in secluded sites, which cannot be representative of all possible conditions found in natural habitats. Moreover, genetic contamination of wild fish by a transgene is not equivalent to gene transfer between varieties or species, because transgenes are proprietary items that can be patented (USA Patent and Trademark Office #5545808 for transgenic salmonid fish expressing exogenous salmonid GH; see also Patent EP 0578 653 B1, granted in 2001 by the European Community to the Canadian

company Seabright for its GH-transgenic Atlantic salmon), and are covered by property rights, licensing agreements and liability for caused damages. This poses unprecedented responsibility for unforeseen harm, in a similar way that newly patented drugs do. For this reason, fertile transgenic fish are not a useful invention, because the potential producers of such fish would be obliged not only to demonstrate their ecosystemic equivalence with non-transgenic fish, but also to account for future unforeseen harm.

Therefore, only sterile transgenic fish, with no reproductive capacity whatsoever, may have a future in the aquaculture of transgenics, whether or not they are growth-enhanced or disease resistant or more efficient in food conversion, because they are likely to inflict only reversible ecosystemic disturbances. Hence, environmental compatibility and company profitability are not always contrasting factors, as often surmised (Stokstad, 2002).

An integration of triploidy induction and transgenic technologies for fishes was first proposed by Thorgaard et al. (1992). The first report on the effects of induced triploidy on growth-enhanced transgenic Nile tilapia was published by Razak et al. (1999). However, less than 70% of the fish grown from heat-shocked fertilised eggs were found to be triploids, so the issue of achieving 100% sterility was not addressed. They noticed that triploid transgenic tilapia grew less than transgenic diploids, but more than non-transgenic diploids. At adulthood, ovaries were non-functional, while testes produced some sperm. Owing to the current level of effectiveness of standard triploidisation techniques, ensuring 100% sterility by this approach is deemed next to impossible (Lee and Donaldson, 2001; Stokstad, 2002; Logar and Pollock, 2005). This may explain why the Food and Drug Administration has not yet granted approval to AquaBounty Farms in Waltham,

Massachusetts, USA, which is the only company that filed, in 1996, for approval to commercially produce its patented AquAdvantage salmon, a fast-growing GH-transgenic Atlantic salmon, despite the claim that it would be sterile (Fletcher et al., 2001). Recently, Farahmand et al. (2008) managed to induce tetraploidy (by applying a multiple heat shock protocol) in growth-enhanced transgenic tilapia, as assessed by karyotyping and beta-galactosidase expression analysis of embryos. Grown fish, however, exhibited different degrees of mosaicism and failed to produce triploids after crossing the tetraploids with regular diploids.

Physical containment in closed circulation systems would be practical only to confine transgenic broodstocks or to carry out experiments, but not for the grow out of fertile transgenic fish because escape must be totally prevented even in case of disasters, like flooding, or because of unauthorized intrusions (MacLean and Laight, 2000). A promising alternative could be to combine triploidy induction with hybridization to progress towards 100% sterility. One such approach was proposed in the work by Nam et al. (2004a,b) who compared fertility and growth of diploid and cold-shocked triploid hybrids, obtained from the crosses between female cyprinid loach and either GH-autotransgenic or non-transgenic male mud loaches, with respect to diploid GH-autotransgenic and non-transgenic mud loaches. Although transgenic mud loaches grew more and converted food better than either type of transgenic hybrids, the transgenic hybrids themselves were largely superior to the non-transgenic fish. More importantly, while diploid hybrids showed reduced fertility, complete sterility was observed in all allotriploids. Nevertheless, since the incidence of triploidy was at least 97%, but not 100%, and diploid hybrids were fertile, full transgenic containment could not be attained in this particular trial.

A definite improvement would be to replace proximal hybrids between closely related species, which can retain some fertility, with hybrids from systematically distant species, like in intergeneric hybridisation, which are totally and permanently sterile owing to unsuccessful pairing of chromosomes. As noted above, some of these hybrids have been adopted for aquaculture, and others appear to be good candidates. This is the case of intergeneric hybrids within the family Sparidae that display greater growth than the parental species, like the cross of female red sea bream x male dentex, *Dentex dentex*, or the reciprocal hybrid (Colombo et al., 1997). Hybrids of female gilthead sea bream x male red sea bream, obtained in both diploid and triploid conditions, were also found to be completely sterile (Gorshkov et al., 2002). An alternative strategy is the application of interploid crossing, mating tetraploid with diploid fish to generate all-triploid progeny (section 5.1.1), though few teleost species are compatible with this ploidy status.

A disconcerting point to be considered is the occurrence of fertile triploids among older fish, which is suggested to be due to unilateral segregation of unpaired chromosomes at meiosis (Arai, 2001; Lee and Donaldson, 2001). The mechanism underlying this phenomenon is undetermined, but it can compromise the safe containment of transgenic fish. For this reason, allotriploids produced from species that can give rise to sterile diploid hybrids secure a better safeguard, because they are under double sterilisation constraints.

In conclusion, there is the possibility through further research effort and private investment to develop the production of transgenic fish that are both nutritionally safe and ecosystem-compatible and sustainable, particularly in the case of non-carnivorous species. Thus, the induction of triploidy can be an option for the containment of GMOs, but

transgenic approaches are also being explored for the same purpose (Wong et al., 2007; 2008).

On the other hand, transgenics can improve the food balance in poor countries under heavy demographic pressure. Fast-growing GH-transgenic fish first appeared in Asia (Zhu et al., 1985). Thus, given the importance of fish culture and the rate of population growth in that continent, where research on transgenesis and polyploidisation is still very active, it will not be surprising if the transition towards the culture of transgenics. takes place there. Polyploidisation may help towards this progress.

9.4. Summary of prospects for the potential use of triploids to limit the genetic impact of escapees on wild populations

For non-GMOs, triploidy is an appropriate method to considerably reduce or eliminate the genetic impact of farmed escapees on wild populations. If autotriploidy is chosen to induce functional sterility in a given species, whether fish or shellfish, current knowledge suggests that, as a precautionary measure, at least two full consecutive reproductive cycles should be monitored to confirm the absence of gamete production. If sterility for biocontainment is found to be necessary for species currently produced and for which commercial triploidisation protocols are not available (cod, meagre, soles, sturgeons, mussels, pectinids, abalone, etc.), these should be developed. Moreover, the use of triploid farming to limit the genetic impact of escapees will be best achieved by constructive interaction between all stakeholders (farmers, anglers, consumers, welfare and environmental groups).

On the other hand, the fact that some autotriploids can produce gametes capable of activation and/or fertilisation, even if this phenomenon appears to be rare and species-specific, hampers the prospect of employing induced autotriploidy as a sole precautionary requirement for the biosafety of GMOs, where 100% biocontainment is required. Otherwise, a reliable method is required for the low-cost, high-throughput, high-efficiency individual verification of ploidy and sterility. In this context, of interest are terminator transgenes that are lethally activated only at the beginning of ovarian maturation, as the construct encoding thiaminase driven by vitellogenin promoter proposed by Devlin et al. (2006). Such an estrogen-sensitive transgene would induce thiamine deficiency and compromise the viability of the few diploid females residual in all-female triploid stocks at the onset of follicular estrogen secretion, unless diploid females are supplied with excess vitamin to propagate the transgenic broodstock.

10. Conclusions

Triploids are requested by many organizations for a variety of reasons and although the basic methods are now well established for their production these need optimising for new species and there are many aspects of triploid biology that remain uncertain. Further research is therefore needed on the induction and biology of triploids in many species in order to consistently achieve high performing sterile triploids for the aquaculture industry.

Further research is required into the biology of triploids, particularly about their growth performance, organoleptic properties and economic profitability in different types of farming environments such as ponds (carp, European catfish), sea cages (European sea bass, gilthead sea bream, and cod), concrete raceways (European sea bass, gilthead sea

bream, turbot, halibut, freshwater salmonids, sturgeon), recirculated systems (marine fishes) and suspended or rack culture (shellfish). In particular, knowledge on the physiology and gene regulation in triploids (hormonal and immune status, functional genomics) reared under optimal or sub-optimal farming conditions should be improved. Further, a better understanding of the mechanisms by which triploidy affects gametogenesis and reproduction is needed.

Of particular importance are investigations into the fitness of induced sterile triploids in the natural environment. Knowledge of relevant traits such as survival, competition for resources, reproductive performance and behaviour in male and female triploids of different species is required to assist with the management of the ecological impact of their accidental escape or deliberate stocking into the wild. To this end, the ecological impact of the escape of small numbers of triploids from farms into the wild and the potential effects of restocking with large numbers of triploids should be evaluated.

Tetraploids are difficult to produce and require specialised containment. Thus, further research on their production is essential. Despite this inconvenience, By crossing with diploids they offer the most effective method to produce 100% triploids and they are therefore a vital resource. Indeed, for GMO containment purposes, 100% sterile triploids are essential and tools for assessing both sterility and accurate ploidy in such organisms must be developed and optimised.

In addition to pure and applied biological research into ploidy manipulation in aquaculture, significant effort should go into public information strategies including web sites, dedicated workshops, labelling, marketing approaches, etc., in order to disseminate

1942 the benefits and possible risks of triploidy. Public bodies have an important role to provide stakeholders with sound scientific results and to facilitate public perception of the interest
1944 of this genotype for the benefit of the aquaculture production sector and for the preservation of the biodiversity. In addition to clarifying the non-GMO status of
1946 polyploids, consumer education programs could use examples of the importance of polyploids in current and past agriculture.

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Acknowledgments

1950

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Table 1. Representative range of values for the main treatment variables involved in the induction of triploidy in fish and shellfish according to type of shock

Animals	Type of shock	Timing[1]	Intensity	Duration
Fish	Pressure	2-7 min in warmwater sp. 15-20 min in coldwater sp.	62 MPa (range: 58-85 MPa)	2-6 min
	Cold	2-7 min in warmwater sp. 15-20 min in coldwater sp.	-1 to 4°C in temperate or warmwater sp.	2-20 min (most cases). 35 min-3 h in coldwater sp.
	Heat	2-7 min in warmwater sp. 15-20 min in coldwater sp.	24-32°C in coldwater sp. 34-41°C in temperate or warmwater sp.	10-25 min in coldwater sp. 45 s – 3.5 min in temperate or warmwater sp.
	Chemical	Temperature-dependant. Just before extrusion of either the 1st or, most commonly, the 2nd polar body. *e.g.*, Pacific oyster at 20°C: 1st pb at 15 min; 2nd pb at 40 min	Cytochalasin B: 0.1-1.0 mg l^{-1} seawater. 6-dimethylaminopurine (6-DMAP): 20-60 mg l^{-1} seawater, final concentration 300 µM	15-20 min
Shellfish				
	Pressure	As above	~60 MPa	10-15 min
	Cold	As above	0-5°C	15-20 min
	Heat	As above	25-38°C	15-20 min

[1]Time after fertilization.

3488 **Table 2.** A selection of key studies on tetraploid production in fish and shellfish.

Species	Methods	Results	References
Fish			
Rainbow trout, *Oncorhynchus mykiss*	Pressure shock and use of inactivated sperm and allo-spermatozoa	Auto- and allo-4N and 2N gynogens produced	Chourrout (1982) Chourrout (1984)
	Thermal shock at 1st cleavage and use of inactivated sperm.	2N gynogens but no 4N in controls	Purdom et al. (1985)
	4N sire x 2N dam with thermal shock at MII	2nd generation 4N offspring produced with better growth and survival than 1st generation 4N	Chourrout et al. (1986)
	4N sire x 2N dam with thermal shock at MII	2nd generation 4N offspring produced	Blanc et al. (1987)
	4N males used to make 3N offspring and 4N females used to make 2N gynogens	Gene segregation studied: preferential pairing of homologous chromosomes	Diter et al. (1988)
	Pressure at 1st cleavage	Significant variation in first cleavage interval (FCI) between populations of fish. Best to assess FCI before induction in individual fish	Hershberger and Hostuttler, 2007
Channel catfish, *Ictalurus punctatus*	Thermal shock at 1st cleavage	62% 4N	Bidwell et al. (1985)
	Pressure shock at MII and 1st cleavage. Use of allo-spermatozoa and inactivated sperm	Up to 4% allotetraploids from targeting 1st cleavage.	Goudie et al. (1995)
African catfish, *Clarias gariepinus*	Thermal shock at 1st cleavage and use of allo-inactivated sperm	2N gynogens with allo-inactivated sperm (6.3%) and 4N with auto-inactivated sperm (9.2%)	Varadi et al. (1999)
Stinging catfish, *Heteropheustes fossilis*	Thermal shock at 1st cleavage. Variation in duration and temperature	Up to 40% 4N produced, but failed to survive until 1st feeding.	Haniffa et al. (2004)
Tilapia, *Oreochromis aureus*	Cold shock at 1st cleavage Variation in timing of shock	4N production optimised (25%) at 90 min post fertilisation	Don and Avtalion (1988)
Mud loach, *Misgurnus mizolepis*	Thermal shock at 1st cleavage. Variation in temperature.	Up to 56% 4N in best treatment. Early mortality, but many 4N adults produced.	Nam et al. (2004b)

Common carp, *Cyprinus carpio*	Thermal shock at 1st cleavage	92-100% 4N larvae, but high mortalities during development. At 2 months only two 4N fish out of 31 survivors.	Cherfas et al. (1993)
Common carp, *Cyprinus carpio* x Red crucian carp, *Carassius auratus*	Crossing of F2 diploids	100% allo-4N in all generations from F3-F8	Liu et al. (2001; 2008)
Indian carp rohu, *Labeo rohita*	Thermal shock at 1st cleavage	70% 4N	Reddy et al. (1990)
Grass carp, *Ctenopharyngodon idella*	Thermal shock and pressure shock at 1st cleavage and/or in multiple cell zygotes. Variation in timing	mean 62.5% 4N with pressure; 0 – 100% 4N with thermal. 4N larvae died till day 50, aneuploids and 2N-4N mosaics noted.	Cassani et al. (1993)
	Thermal shock at 1st cleavage. Variation in timing	up to 42% 4N larvae	Zhang et al. (1993)
Bighead carp, *Aristichthys nobilis*	Thermal shock at 1st cleavage. Variation in timing	56% 4N produced but timing of heat shock is critical. Lower hatchability and higher abnormalities in 4N cohort	Hong (1990)
Catla, *Catla catla*	Thermal shock at 1st cleavage	65% 4N	Reddy et al. (1990)
Tench, *Tinca tinca*	Thermal shock and pressure shock at 1st cleavage	At fry stage thermal, 42% 4N; pressure: 62% 4N. Good survival to adult.	Flajšhans et al. (1993)
Blunt snout bream, *Megalbrama amblycephala*	Thermal shock at 1st cleavage. Variation in timing and temperature	Up to 6.3% 4N at one year. Most males matured at 2 yr, most females at 3-4 yr	Zou et al. (2004)
Masu salmon, *Oncorhynchus masou*	Pressure shock at 1st cleavage and use of inactivated sperm.	All 4N died around hatching time (34 dpf.). 2N gynogens survived beyond 55 dpf	Sakao et al. (2006)
European sea bass, *Dicentrarchus labrax*	Pressure shock at 1st cleavage	6-25% survival of 4N at hatching	Barbaro et al. (1998)
	Use of inactivated sperm with pressure shock at 2nd pb and 1st cleavage.	Very few 4N in most batches, one with 94% 4N	Peruzzi and Chatain (2003)
	Pressure shock at 1st cleavage. Variation in intensity and timing. Use of inactivated sperm	75-94% 4Nin11 day old larvae reduced to 4% 4N in 46 day old fry	Francescon et al. (2004)
	Pressure shock at 1st cleavage. Use of inactivated sperm	4N hatched larvae from control	Bertotto et al. (2005)

Yellow perch *Perca flavescens*	Pressure shock at 1st cleavage	80% survival at 7 day old of 100% 4N larvae. Some juvenile tetraploids produced	Malison et al. (1993a)
Shellfish			
Pacific oyster, *Crassostrea gigas*	Eggs from 3N crossed with N sperm and suppression of 1st pb.	First viable 4N from a bivalve mollusc	Guo and Allen (1994a)
Pearl oyster, *Pinctada martensii*	Eggs from 3N crossed with 1N sperm and suppression of 1st pb.	Mostly 2N, 3N or XN; 2 individuals 4N at 1 yr old	He et al. (2000)
American oyster, *Crassostrea virginica*	Eggs from 3N crossed with 1N sperm and suppression of 1st pb.	>4,000 spat from 13 trials, but at 5 months old, 10% changed to 3N/4Nmosaics	Guo et al. (2002)
Suminoe oyster, *Crassostrea ariakensis*	Eggs from 3N crossed with 1N sperm and suppression of 1st pb.	Several thousand spat from 21 trials. Larvae much larger than 2N at setting	Allen et al. (2003)
Sydney rock oyster, *Saccostrea glomerata*	Various methods	Large numbers of 4N larvae produced but few survived.	Nell (2002)
Catarina scallop, *Argopecten ventricosus*	Eggs from 3N crossed with 1N sperm and suppression of 1st pb.	6 scallops survived to juvenile stage, 5 were 4N, the remaining scallop was mosaic 4N/2N	Maldonado et al. (2003)
Mediterranean mussel, *Mytilus galloprovincialis*	Suppression of both 1st pb and 2nd pb	17% 4N of one month old spat	Scarpa et al. (1993)
Manila clam, *Tapes philippinarum*	Suppression of 1st pb and of 1st cleavage using CB	64% 4N (1st pb) and 28% 4N (1st cleavage) at 6 hr. None survived to spat stage	Diter and Dufy (1990)
	Suppression of 2nd pb intended to make triploids	A few individual 4N adults within 3N progeny	Allen et al. (1994)
Zhikong scallop, *Chlamys farreri*	Suppression of 1st pb using CB	Up to 2% of spat 4N, but no juveniles	Yang et al. (2000)
Dwarf surf clam, *Mulinia lateralis*	Thermal shock at 1st or 2nd cleavage divisions	44-82% 4N, but non-viable beyond larval stage	Yang and Guo (2006)
	Suppression of 1st pb using CB. Varied temperature.	Up to 2% of spat 4N, but no juveniles	Peruzzi and Guo (2002)
	Suppression of 1st pb using CB. Varied duration.	Up to 0.6% 4N in 1-2 months old juveniles	Yang and Guo (2004)
Kurama shrimp, *Marsupenaeus japonicus*	Suppression of 1st cleavage by thermal (heat and cold) and chemical (6-DMAP) shock	Up to 98% 4N embryos from thermal shock but none developed to larvae. No 4Nembryos from chemical treatment	Sellars et al. (2006a)

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Abbreviations: CB, cytochalasin-B; dpf, days-post fertilisation; MII, meiosis II; pb, polar
3492 body; 1N, haploid; 2N, diploid; 4N, tetraploid; 6-DMAP, 6-dimethylaminopurine.

3494 **Table 3.** Effects of induced triploidy on growth performance and gonadal development in some commercially important fish, shellfish and crustaceans

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Species	Growth	Reproduction	References
Fish			
Atlantic salmon, *Salmo salar*	3N = 2N in juveniles; 3N > 2N in adults	Full gonadal sterility in females; aneuploid sperm in males	Benfey and Sutterlin (1984b) O'Flynn et al. (1997)
Rainbow trout, *Oncorhynchus mykiss*	3N < 2N immature 3N > 2N mature	Retarded ovarian development, rare presence of oocytes. Males produced small amount of aneuploid sperm capable of fertilisation.	Solar et al. (1984) Chourrout et al. (1986) Lincoln and Scott (1984)
Brook trout, *Salvelinus fontinalis*	3N = 2N immature 3N > 2N mature		Boulanger, 1991
Coho salmon, *Oncorhynchus kisutch*	3N = 2N in juveniles 3N < 2N in adults	Full gonadal sterility in females; retarded gonad growth in males at 30 mo.	Withler et al. (1995) Johnson et al. (1986)
Tilapia, *Oreochromis mossambicus*	3N < 2N up to 8 months		Penman et al. (1987)
Tilapia, *Oreochromis aureus*	3N = 2N up to 6 months 3N = 2N mature		Don and Avatlion (1986) Byamungu et al. (2001)
Tilapia, *Oreochromis niloticus*	3N = 2N immature 3N > 2N mature		Brämick et al (1995) Pechsiri and Yakupitiyage (2005)
Channel catfish, *Ictalurus punctatus*	3N = 2N in juveniles; 3N > 2N in adults		Wolters et al. (1982)
European catfish, *Silurus glanis*	3N < 2N up to 5 months 3N > 2N mature	Retarded ovarian development	Linhart et al. (2001) Krasznai and Márián (1986)
Chinese catfish, *Clarias fuscus*	3N > 2N at 6 months		Qin et al. (1998)
Asian catfish, *Clarias macrocephalus*	3N > 2N at 8 months		Fast et al. (1995)
African catfish, *Clarias gariepinus*	3N = 2N at 7 months		Henken et al. (1987)
Indian catfish, *Heteropneustes fossilis*	3N > 2N mature		Tiwary et al. (1997)

Tench, *Tinca tinca*	3N > 2N in adults	Full gonadal sterility in females; aneuploid/euploid sperm in males	Flajšhans et al. (1993) Flajšhans (1997) Linhart et al. (2006)
Grass carp, *Ctenopharyngodon idella*	3N ≤ 2N immature	Production of aneuploid eggs and sperm, partly capable of fertilization	Cassani and Caton (1986) Goudie (1988) Van Eenennaam et al. (1990)
Common carp, *Cyprinus carpio*	3N < 2N in juveniles; 3N < 2N in adults	Occasional ovarian development, aneuploid sperm	Cherfas et al. (1994; 1995b)
Perch, *Perca flavescens*	3N < 2N in juveniles		Malison et al. (1993b)
Mud loach, *Misgurnus mizolepis*	3N = 2N at 9 months		Kim et al. (1994)
Cyprinid loach, *Misgurnus anguillicaudatus*	3N < 2N immature 3N > 2N at 1 year	Full gonadal sterility in both sexes. Females produced euploid eggs.	Suzuki et al. (1985) Matsubara et al. (1995)
European sea bass, *Dicentrarchus labrax*	3N = 2N up to 2 years 3N < 2N in adults up to 4 years	Full gonadal sterility in both sexes	Felip et al. (1999; 2001b) Peruzzi et al. (2004)
Turbot, *Scophthalmus maximus*	3N = 2N during first year 3N > 2N after two years	Full gonadal sterility in both sexes	Cal et al. (2006)
Shi drum, *Umbrina cirrosa*	3N < 2N in adults		Segato et al. (2006)
Red seabream, *Pagrus major*	3N = 2N up to 10 months	Males produced euploid sperm up to the heptaploid level.	Sugama et al. (1992) Kawamura et al. (1995)
Gilthead sea bream, *Sparus aurata*	3N = 2N up to 17 months (all male)	3N remained male when diploids sex-changed to female No spermatozoa	Haffray et al. (2005)
Plaice, *Pleuronectes platessa* x European flounder, *Platichtys flesus*, hybrids	3N = 2N in juveniles 3N ≥ 2N in mature		Purdom (1972) Lincoln (1981b)
Japanese flounder ("hirame"), *Paralichthys olivaceus*	3N = 2N in immature and mature		Tabata (1991) in Arai (2001)
Yellowtail flounder, *Limanda ferruginea*		Reduced gonadal development in both sexes but presence of vitellogenic oocytes, sperm partly capable of fertilization	Manning et al. (2004)

Ayu or sweet fish, *Plecoglossus altivelis*	3N = 2N at 6 months	Reduced gonadal development in both sexes, rare presence of vitellogenic oocytes or spermatids.	Lee et al. (1998) Ueno et al. (1986)
Fighting fish, *Betta splendens*	3N = 2N immature		Kavumpurath and Pandian (1992)
Shellfish			
Common mussel, *Mytilus edulis*	3N = 2N up to 4 months 3N > 2N afterwards Growth difference more pronounced in high food environment		Beaumont and Kelly (1989) Beaumont (2000) Brake et al. (2004)
Mediterranean mussel, *Mytilus galloprovincialis*	3N = 2N at 14 months	Reduced gonadal development and maturation	Davis (1997)
Pacific oyster, *Crassostrea gigas*	3N = 2N first year 3N > 2N second year		Allen and Downing (1986) Boudry et al. (1998) Wang et al. (2003)
American oyster, *Crassotrea virginica*	3N = 2N up to 8 months 3N (MI) > 3N (meiosis MII) = 2N at 2 years 3N > 2N as adult		Stanley et al. (1984) Barber and Mann (1991)
Sydney rock oyster, *Saccostrea commercialis*	3N > 2N but similar Scope For Growth	Sterility in both sexes: Spermatocyte in males Secondary oocyte in females	Cox et al. (1996) Kesarcodi-Watson et al. (2001)
Japanese pearl oyster, *Pinctada fucata martensii*	3N (meiosis I) > 3N (meiosis II) = 2N in juveniles 3N (meiosis I) = 3N (meiosis II) > 2N in adults	Some spermatozoa detected in males	Jiang et al. (1993) Komaru and Wada (1990)
Scallop, *Chlamys nobilis*	3N = 2N up to 9 months 3N > 2N at 14 months		Komaru and Wada (1989)
Chinese scallop, *Chlamys farreri*	3N = 2N at 60 and 450 days (3N > 2N in adductor muscles)		Yang et al. (2000)
Lion-paw scallop *Nodipecten subnodosus*	3N = 2N	Gonadosomatic index in 2N > 3N. More 22:6n-3 PUFA in 3N	Racotta et al. (2008)
Bay scallop, *Argopecten irradians*	3N > 2N		Tabarini (1984)

Catarina scallop, *Argopecten ventricosus*	3N > 2N		Ruiz-Verdugo et al. (2000)
Great scallop, *Pecten maximus*	3N > 2N		Beaumont, 2000
Hard-shelled Clam, *Mercenaria mercenaria*	3N = 2N up to 27 months 3N > 2N at 47 months		Eversole et al. (1996)
Dwarf surf clam, *Mulinia lateralis*	3N > 2N		Guo and Allen, 1994b
Manila clam, *Tapes philippinarum*	3N = 2N up to sexual maturity 3N ≤ 2N afterwards		Ekaratne and Davenport (1993)
Abalone, *Haliotis discus reeve*	3N = 2N up to 14 months 3N > 2N at 28 months		Chen et al. (2002)
Pacific Abalone, *Haliotis discus hannai*	3N = 2N first year 3N > 2N second year 3N > 2N up to 4 months		Sun et al. (1992) Zhang et al. (1998)
Abalone, *Haliotis diversicolor aquatilis*	3N > 2 n at 7 months		Yan and Chen (2002)
Soft shell clam, *Mya arenaria*	3N = 2N in juveniles 3N > 2N second year		Mason et al. (1988)
Crustaceans			
Chinese shrimp, *Fenneropenaeus (Penaeus) chinensis*	3N = 2N immature stage, specific growth rate of 3N lower than 2N 3N > 2N mature stage	Reduced gonadal development in females but not in males. Few developed oocytes in females and only spermatids in testis	Xiang et al. (2006) Zhang et al. (2008)

3498 Abbreviations: MI, meiosis I; MII, meiosis II; 2N, diploid; 3N, triploid.

3500 **Table 4.** Growth and survival of immature triploid (3n) and diploid (2n) fishes observed in common garden experiments

Species	Rearing conditions	Survival 3n / 2n	Growth 3n / 2n	References
Rainbow trout, *Oncorhynchus mykiss*	Separate		=	Lincoln and Bye (1987)
	Mixed		<	
	Mixed	<	<	Thorgaard et al. (1982)
	Separate up to 12 months	<	<	Quillet et al. (1988)
	Mixed afterwards	=	<	
	Freshwater culture Mixed, hot temperature	<	<	Ojolick et al. (1995)
	Seawater culture			
Atlantic salmon, *Salmo salar*	Separate up to 9 months	<	=	Quillet and Gagnon (1990)
	Mixed afterwards	<	<	
	4 to 8 months : separate	<	>	Galbreath et al. (1994)
	8 to 17 months : mixed		<	
	Mixed	=	=	McGeachy et al. (1995)
Grass carp, *Ctenopharyngodon idella*	Separate, optimal conditions		=	Cassani et al. (1984)
	Mixed		<	
Common carp, *Cyprinus carpio*	Mixed	<	<	Cherfas et al. (1994)
	Mixed		=	Gervai et al. (1980)
Tench, *Tinca tinca*	Mixed	=	>	Flajšhans et al. (1993)
	Separate up to 12 months	<	=	Flajšhans et al. (2004)
	Mixed afterwards	=	>	
Pacific salmon, *Oncorhynchus kisutch*	Mixed Adverse conditions	<	<	Utter et al. (1983)
	Mixed Optimal conditions	<	=	Johnson et al. (1986)
African catfish, *Clarias gariepinus*	Separate		=	Henken et al. (1987)
	Mixed		=	
European catfish, *Silurus glanis*	Mixed up to 5 months	<	<	Linhart et al. (2001)
Cyprinid loach, *Misgurnus anguillicaudatus*	Mixed		<	Suzuki et al. (1985)

3502

Table 5. Summary on the advantages and disadvantages of triploidy induction for the aquaculture of fish and shellfish

Advantages	Disadvantages
Increased post-pubertal body growth	Increased initial mortality
Can reduce counterproductive effects of sexual maturation	Increased deformities
Can reduce reproductive interaction	May decrease prepubertal growth
Avoid genetic impact of escapees	Difficulty of integration with selection programs
Year-round marketability of triploid oysters	Consumer acceptance
Enables sterile triploid hybrids	Reversion of ploidy in Pacific oysters

3508 **Table 6.** Summary of studies reporting the behaviour of sterile fish in the wild

Species	Method to induce sterility	Main effects	References
Coho salmon, *Oncorhynchus kisutch*	Hormonal treatment	Lack of homing behaviour. Males display sexual behaviour in the wild	Solar et al. (1986)
		Display site fidelity	Bridger et al. (2001)
Rainbow trout, *Oncorhynchus mykiss*	Induction of triploidy	Same capture rate by anglers	Dillon et al. (2000)
		Same poststocking survival	Wagner et al. (2006)
Atlantic salmon, *Salmo salar*	Induction of triploidy	Male triploids migrate; females do not	Wilkins et al. (2001)
	Induction of triploidy	Inability to interbreed among themselves or with wild populations	Cotter et al. (2000)

3510

Figure Legends

Figure 1. Ploidy manipulation in shellfish. Eggs are released at metaphase of Meiosis I. Fertilisation resumes meiosis. Physical or chemical shock applied during Meiosis I or Meiosis II can suppress cell division, producing triploids by retention of the first (PB1) or second (PB2) polar body. For simplicity, in this hypothetical species 2n = 2. Thus, each bar inside the cell represents one chromosome and overlapping bars indicate the sister chromatids after DNA replication during meiosis I.

Figure 2. Ploidy manipulation in fish. Eggs are released at metaphase of Meiosis II. Fertilization resumes meiosis. Physical or chemical shock applied during Meiosis II or first cleavage can suppress cell division while allowing chromosomal division, producing triploids (Meiosis II suppressed) or tetraploids (first cleavage suppressed). For simplicity, in this hypothetical species 2n = 2. Thus, each bar inside the cell represents one chromosome and overlapping bars indicate the sister chromatids after DNA replication during meiosis I.

Figure 3. Methods commonly used to identify the ploidy level in fish and shellfish. Modified from Piferrer et al. (2007), reproduced with permission.

Figure 4. Production of tetraploid Pacific oysters, *Crassostrea gigas*, from a cross between eggs from a triploid female and spermatozoa from a diploid male with suppression of first polar body (PB1) extrusion. The haploid number for Pacific oyster is 10, and 15 chromosomes are indicated in the triploid egg after meiosis II, which along with the 10 chromosomes provided by the sperm result in an aneuploid embryos (2n = 25).

Here, each bar inside the cell represents an entire haploid complement of 10
3538 chromosomes. Illustration based on Fig. 4 of Guo and Allen (1994a), with modifications.

3540 **Figure 5.** Photographs of gonads of adult diploid and triploid male and female European sea bass, *Dicentrarchus labrax*, showing the characteristic pattern of triploidy effects at
3542 different ages. A and B, 2-year-old fish; C and D, 3-year-old fish; E and F, 4-year-old fish. In each photograph, the testis is in the top and the ovary at the bottom. Modified from
3544 Felip et al. (2001c), with permission.

3546 **Figure 6.** Photomicrographs of ovaries from diploid and triploid female European sea bass. (A) Ovary of a 2-year-old diploid female, containing perinucleolar (po) and
3548 previtellogenic oocytes (pvo). (B) Ovary of a 2-year-old triploid female, exhibiting oogonia and germ cells in early meiotic stages with some perinucleolar and previtellogenic
3550 oocytes. Note the ovarian lamellae. (C, D) Ovaries of a 3-year-old triploid female, exhibiting germ cells in the early meiotic stages: leptotene (lt) and zygotene (zg). Oogonia
3552 are indicated by arrows. (E) Vitellogenic oocytes (vo) of a 4-year-old diploid female. Note the zona radiata (zr), lipid droplets (ly), and protein yolk granule (py). (F) Ovary of a 4-
3554 year-old triploid female with sporadic early vitellogenic oocytes. Bar = 100 μm in A, B and E, F; 20 μm in C; and 10 μm in D. Reproduced from Felip et al. (2001c), with
3556 permission.

3558 **Figure 7.** Photomicrographs of testes from diploid and triploid male European sea bass. (A, C) Testis of 2- and 3-year-old diploid males, respectively, exhibited an active
3560 spermatogenesis and produced sperm. (B, D) Testis of triploid 2- and 3-year-old males, respectively, exhibiting an apparently normal spermatogenesis, but note the absence of

spermatozoa in triploids. Comparison of germ cell size of 4-year-old diploid (E) and triploid (F) males. In triploid males, primary spermatocytes undergo abnormal division (dc). Abbreviations: spermatogonia (arrows), primary spermatocytes (ps), secondary spermatocytes (ss), spermatids (sp), and spermatozoa (sz). Bar = 20 μm in A, B; 50 μm in C, D; and 10 μm in E, F. Reproduced from Felip et al. (2001c), with permission.

Figure 8. Sperm motility and velocity in diploid (2n) and triploid (3n) tench, *Tinca tinca*. (A) 3n fish had a reduced initial number of motile spermatozoa but due to interindividual variability (not shown) differences were no longer significant after 60 s. (B) In contrast, initially similar spermatozoa velocities began to differ 45 s after their activation and onwards, showing higher velocity of spermatozoa of 3n. Figure generated with original data of Linhart et al. (2006).

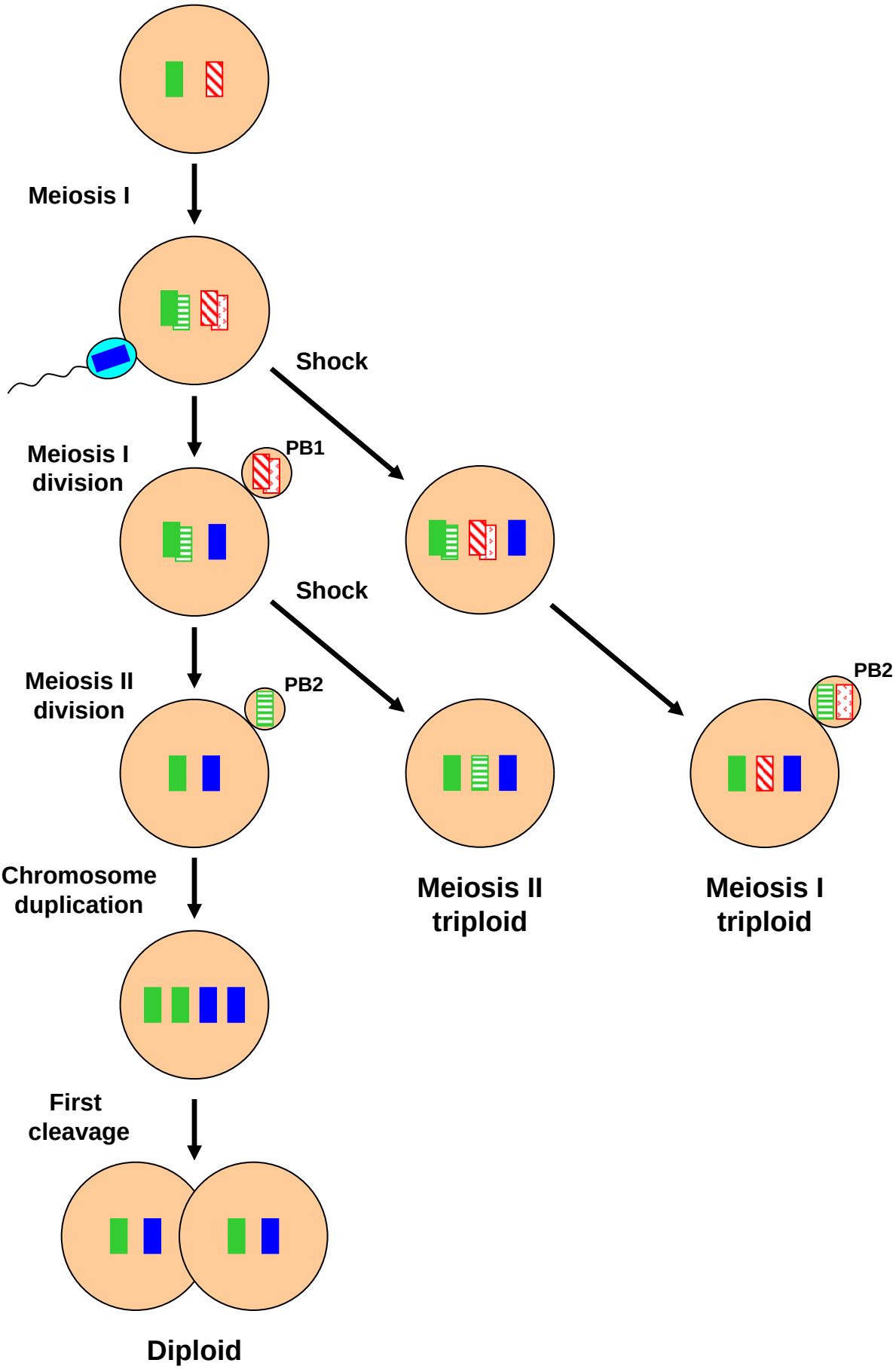
Meiosis I
Shock
Meiosis I
division
PB1
Shock
Meiosis II
division
PB2
PB2
Chromosome
duplication
Meiosis II
triploid
Meiosis I
triploid
First
cleavage
Diploid

Figure 1

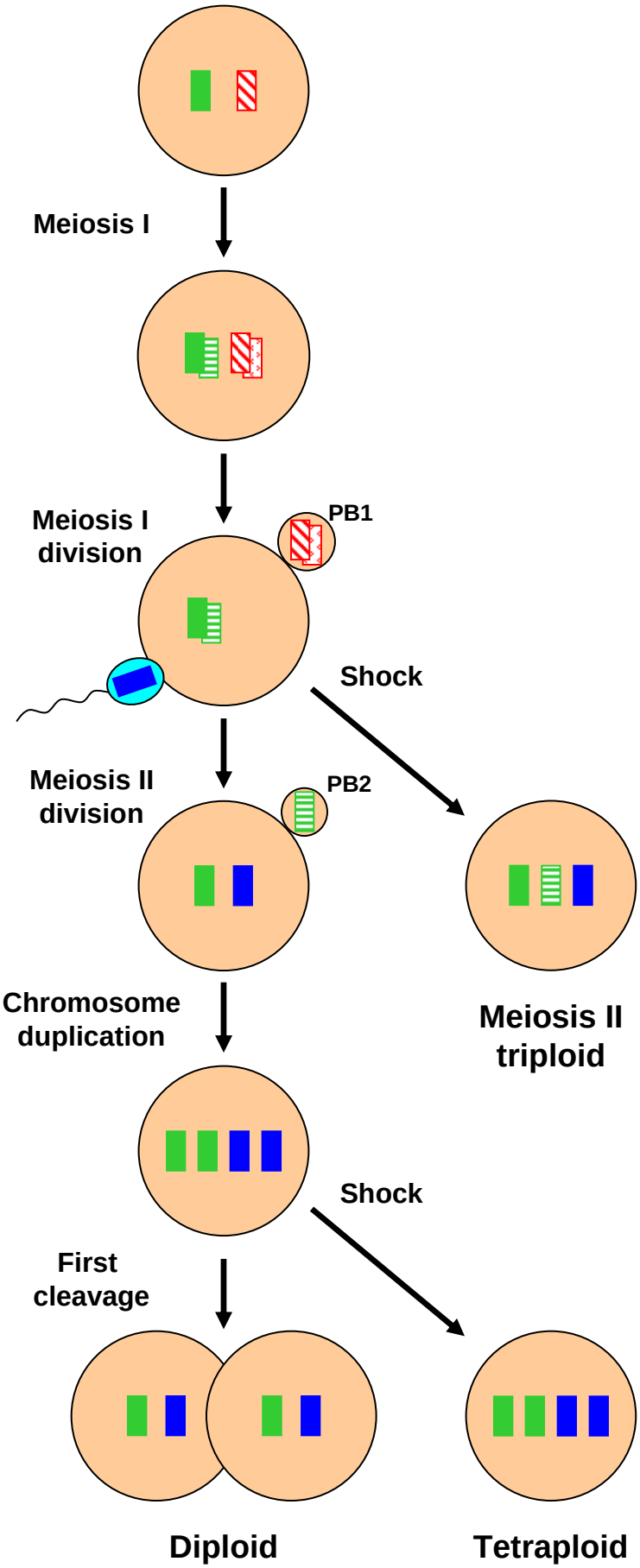
Meiosis I
Meiosis I division
PB1
Shock
Meiosis II division
PB2
Meiosis II triploid
Chromosome duplication
Shock
First cleavage
Diploid
Tetraploid

Figure 2

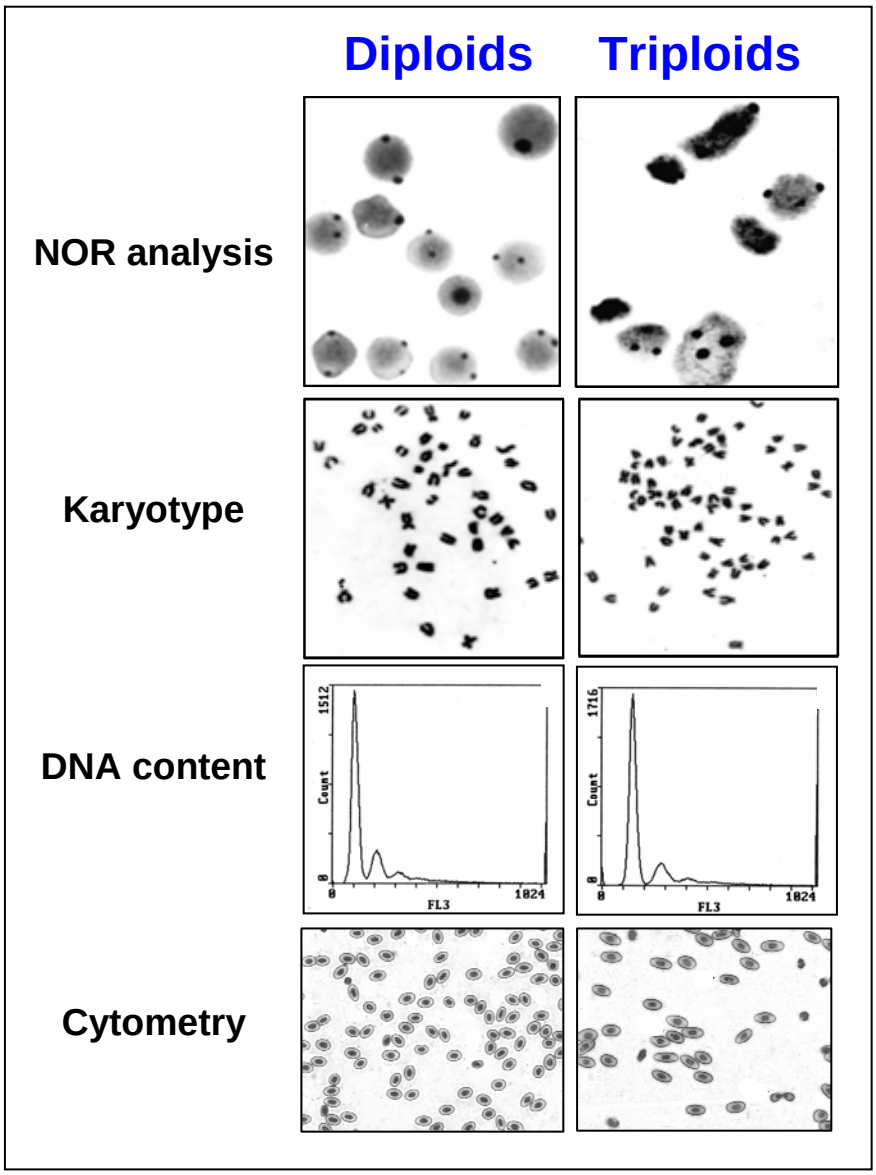
Diploids
Triploids
NOR analysis
Karyotype
DNA content
Cytometry
1512
Count
0
0
FL3
1024
1716
Count
0
0
FL3
1024

Figure 3

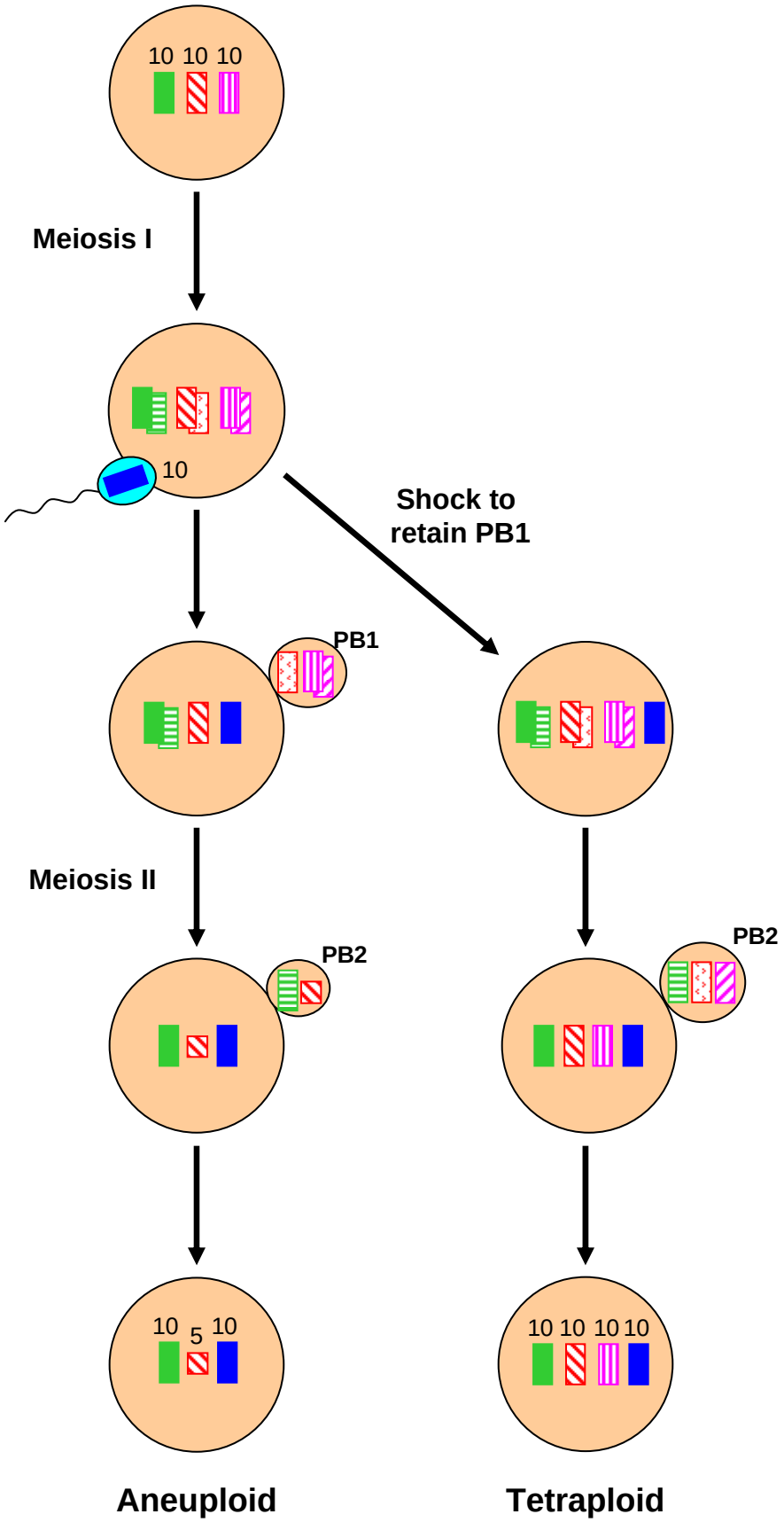
10 10 10
Meiosis I
10
Shock to
retain PB1
PB1
Meiosis II
PB2
PB2
10 5 10
10 10 10 10
Aneuploid
Tetraploid

Figure 4

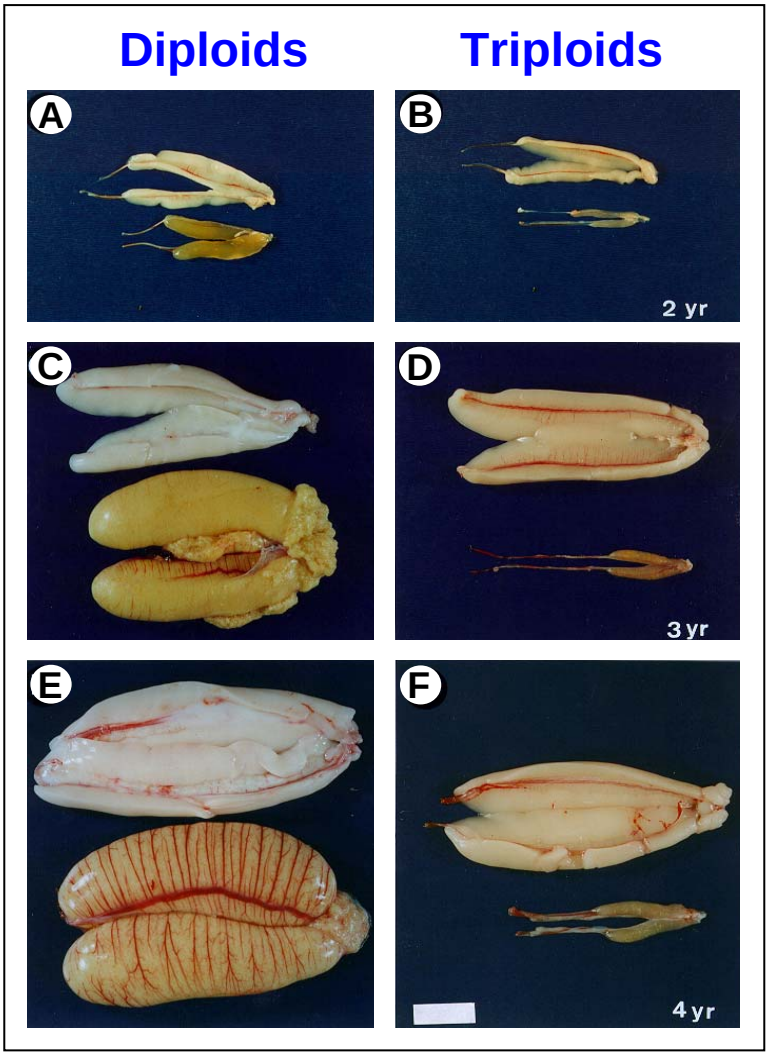

Figure 5

2 years

3 years

4 years

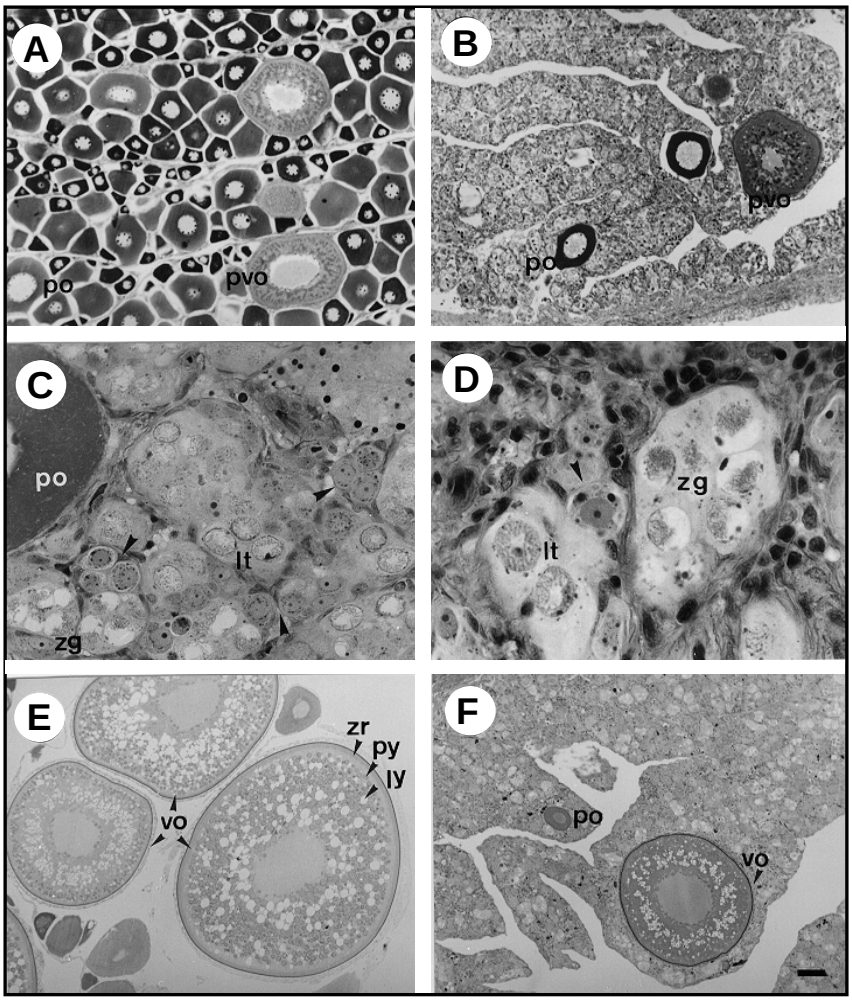

Figure 6

2 years

3 years

4 years

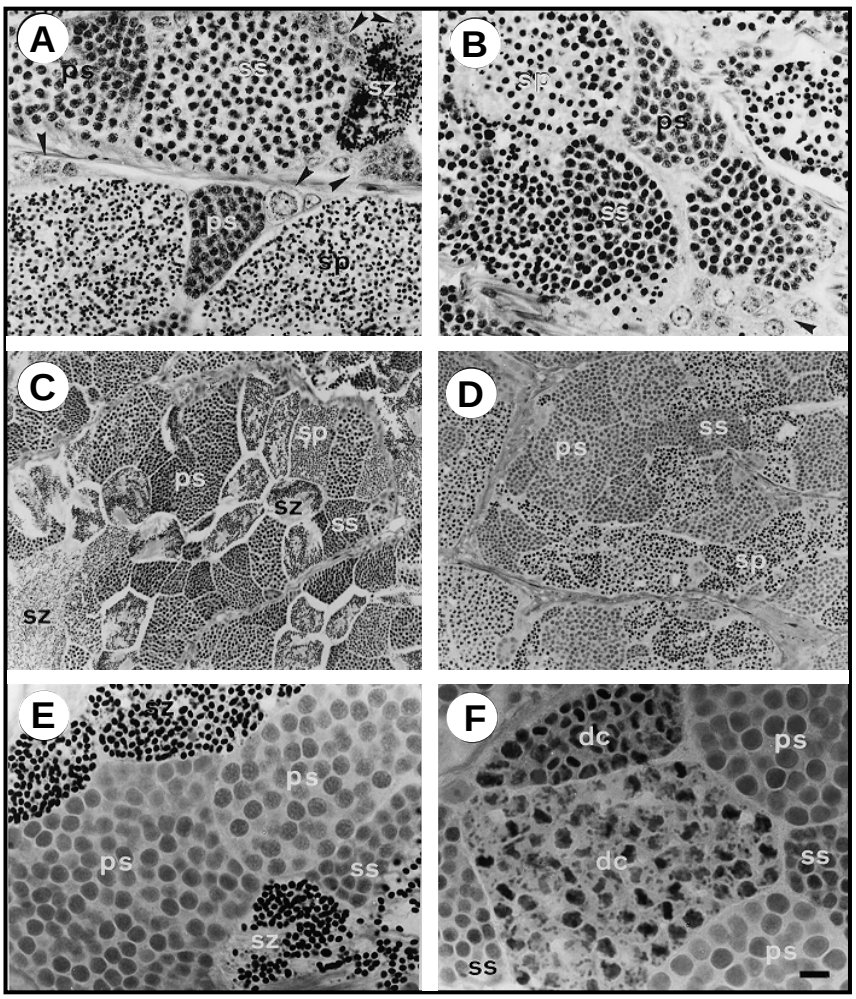

Figure 7

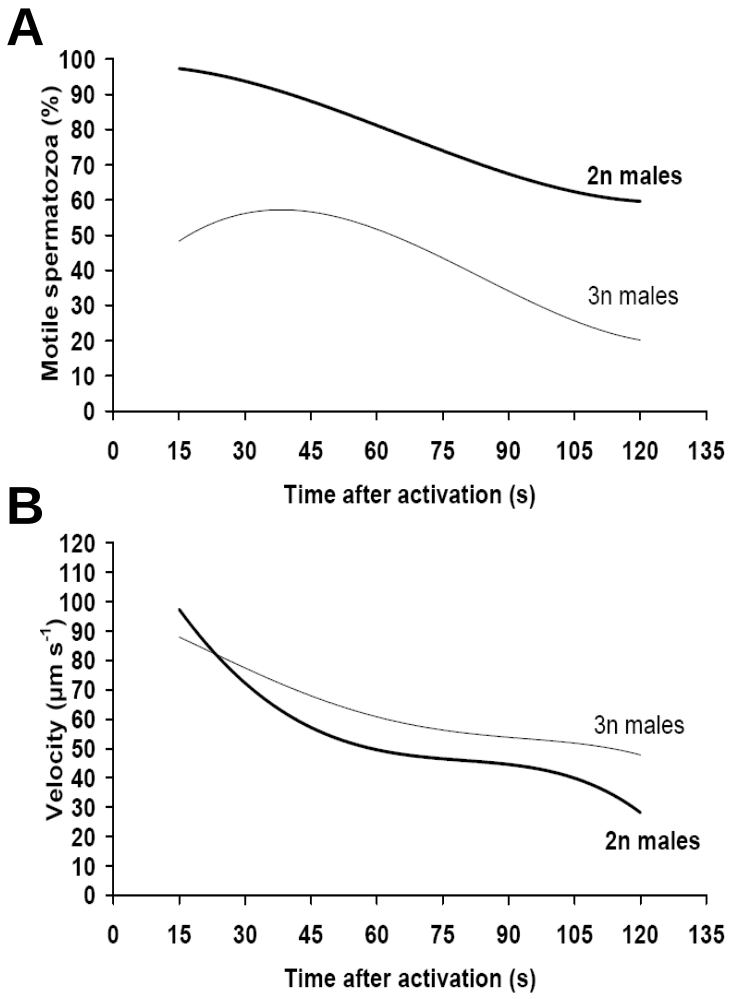
A
Motile spermatozoa (%)
100
90
80
70
60
50
40
30
20
10
0
2n males
3n males
0
15
30
45
60
75
90
105
120
135
Time after activation (s)
B
Velocity (µm s-1)
120
110
100
90
80
70
60
50
40
30
20
10
0
3n males
2n males
0
15
30
45
60
75
90
105
120
135
Time after activation (s)

Figure 8