Chromosomal Manipulation in Fish (Part I)

An organism's ploidy is the number of copies of each chromosome set that it has. Usually gametes have a single set—they are haploid—and two gametes fuse to form a diploid zygote. However, it is viable for organisms to possess three (triploid), four (tetraploid) or more copies of each chromosome, in which case they are known as polyploids. Polyploidy can happen in nature—one example being hexaploidy in wheat - but it can also be induced. Because the eggs of most fish and shellfish are released into water before fertilisation it is relatively easy to access the maturation divisions of the egg and the early divisions of the embryo. Such access allows manipulation to create polyploid embryos and can also be used to produce diploid embryos that contain only maternal chromosomes—gynogens, or only paternal chromosomes - androgens. This is one field of biotechnological development in aquaculture that does not have its origins in agriculture, given the difficulty in accessing the egg in agricultural animals and plants.

The reader's first question might be: What is the point of ploidy manipulation? The short answer is that by manipulating ploidy we can produce sterile, unisex or highly homozygous cohorts of animals. The aquacultural value of such lines will be discussed later in the chapter; let us first consider the basic principles of ploidy manipulation. An outline of the process of meiosis and it will be recalled that there is

an initial reduction division (meiosis I) in which the chromosome number is halved, followed by a mitotic-type division (meiosis II) during which these haploid chromosome sets are copied into two daughter cells each. Thus, in males, four haploid spermatozoa are produced from each diploid germ cell. However, in females, both meiosis I and meiosis II produce daughter cells of such very uneven size that the smalier cells (the polar bodies) often do not complete meiosis, therefore only one haploid egg and two (rarely three) polar bodies are formed from each diploid germ cell. It is not just that fish and shellfish eggs are released into water that makes ploidy manipulation possible, but rather that the meiotic divisions have not been completed when the eggs are spawned. In the case of most molluscs, eggs are released at metaphase of meiosis I. The first polar body has yet to be produced, and an even more convenient feature is that activation by spermatozoa is required before meiosis will proceed. In most fish the eggs are released after meiosis I with the first polar body present, but further development through meiosis II is again dependent on activation by spermatozoa.

We should note that, although there is extertral fertilisation in most fish and molluscs, this is not generally the case in crustacean shellfish nor in gastropods such as the whelk, and that this limits the potential of ploidy manipulation in these groups. Also, among the bivalve molluscs, the flat oysters (e.g. the European native oyster, *Ostrea edulis)* retain their eggs and brood their embryos in the mantle cavity.

The basic idea behind the method of ploidy manipulation is to allow chromosome replication, but prevent cell division. In that way daughter cells have double the number of chromosomes. If we are able to suppress the cytoplasmic division of meiosis I then the resultant single cell will contain all the pairs of chromosomes and no first polar body is formed. What would normally be two haploid cells will be a single diploid cell. If, on the other hand, meiosis I is allowed to proceed as normal and cytoplasmic division in meiosis II is suppressed instead, then the chromosomes normally expelled into the second polar body are retained and a diploid cell again results. Thus, manipulating ploidy at meiosis I or meiosis II makes the egg diploid rather than haploid. Subsequent syngamy with the haploid male pronucleus results in a triploid embryo. In fish, meiosis II is always the division targeted for the production of triploids because meiosis I has been completed before spawning. In molluscs, while it may not initially appear to matter whether the triploids produced come from suppression of meiosis I (MI triploids) or meiosis II (MII triploids), there is a potential effect on heterozygosity, which we will consider later.

Another trick is to allow both meiotic divisions to take place, and to allow the male and female pronuclei to unite in syngamy, but to suppress the first cleavage division of the zygote. This again produces a daughter cell with double the number of chromosomes - a tetraploid. Although tetraploid fish are produced using this approach, it is far less successful in molluscs; alternative methods have been developed for the tetraploidisation of oysters and these will be discussed later. The general details of triploid and tetraploid production.

Production of Polyploidy

So how is the cytoplasmic division of the cell suppressed? Surprisingly simply, it turns out. All that is needed is some kind of physical or chemical shock delivered at the start of division and maintained for a short period. Physical and chemical shocks are very seldom 100% effective and the proportion of eggs that respond depends upon a number of factors - principally shock magnitude and timing. Of the physical shocks, heat and cold are the easiest to administer and consist of a sudden raising or lowering of the temperature by 5-10°C from ambient. Although hot and cold shocks can produce good results in warm- and cold-water fish, hot shocks seem to produce better results in cold-water fish and vice-versa. Another physical shock method commonly used is to place eggs into a pressure chamber and subject them to pressure of up to 9000 lb in $^{-2}$ (= 60 megapascals or 600 bar; normal SCUBA tanks are pressurised to around 3000 lb in^{-2}). Apart from the hazards of working with such a high-pressure vessel, a further problem with this method is that the number of eggs that can be treated at any one time is limited by the volume of the pressure chamber.

Physical shocks seem to work well in fish species, but less well in molluscs. The standard method for cell division suppression in molluscs (principally developed for oysters) is chemical, involving the use of cytochalasin B (CB). CB is a fun extract which apparently inhibits microfilament formation and development of the cleavage furrow, and therefore prevents cells from dividing successfully. As it is not very water-soluble, CB is first dissolved in dimethyl sulphoxide (DMSO) which enables it to be subsequently dissolved in water. In addition, DMS assists the chemical to enter the eggs through the cell wall (DMSO is so highly penetrative that it is also used as a cryoprotectant and as an ingredient in the spray-on pain killers used by athletes). As might be expected by its mode of action, CB is highly toxic to humans and although the amounts used are very small (up to 2 mg l^{-1}) and the application of CB takes place before the

embryo begins to develop, concerns have been expressed about the danger to humans when consuming ploidy-manipulated oysters. Partly as a result of these concerns, the less toxic chemical, 6-dimethylaminopurine (6-DMAP), which is water soluble, has also been developed for use in shellfish ploidy manipulation.

Gynogens And Androgens

The method to produce gynogens involves the destruction of a complete male chromosome set together wi· he division suppression methods used in making triploids and tetraploids. In gynogen production, sperm are treated for a short time with X rays, gamma rays, or, most commonly, by UV irradiation. This effectively breaks up the chromosomes but does not destroy the motility or the ability of such sterile spermatozoa, or sterizoa, to activate eggs. Eggs activated by sterizoa would normally result in haploid embryos and these are invariably nonviable. However, in molluscs, any one of the three divisions—meiosis I, meiosis II or first cleavage—can be suppressed in order to double the chromosome number, while in fish, meiosis II and first cleavage can be targeted to ensure diploidy.. In all cases, the diploid embryos produced contain only the chromosomes of the female parent. Because there is no contribution from male chromosomes inbreeding is high, but the level of inbreeding in gynogens will depend on which division is targeted. Doubling the chromosome number at first cleavage results in homozygosity at every locus in the genome. That is not to say that all the embryos are identical clones, but each individual is homozygous at all of its loci and thus 100% inbred. These mitotically-produced individuals are called mitogynes and, because all loci are homozygous, any deleterious recessive alleles are exposed with consequent reduction in viability. Gynogens produced by suppression of meiosis I or meiosis II are called meiogynes and they will not be entirely inbred because of recombination events occurring during meiosis. We will consider this in more detail later.

Androgens are individuals whose chromosomes are entirely paternal. They can be produced by two methods, both of which require that eggs are irradiated to destroy their chromosomes. The first uses normal haploid sperm to fertilise the irradiated eggs and then the first cleavage division is suppressed to produce a diploid embryo. The second employs diploid sperm—the gonad product of a tetraploid male—to fertilise the irradiated egg and diploid embryos are produced without further treatment.

Recognition of Ploidy

Production of triploids by physical or chemical suppression o

divisions seldom results in 100% triploids and production of tetraploids is even less certain. For this reason, hatcheries need to establish how successful their triploid or tetraploid induction efforts have been. A balance must be struck between the proportion of triploids in a batch and the costs of ensuring triploidy induction. There are a number of ways in which the ploidy of individual fish or shellfish can be identified and the quickest method is to use a flow-cytometer, which measures the amount of DNA-specific stain taken up by nuclei of individual cells in a sample. A graphical display allows calculation of the proportion of individuals in the sample that are triploid and the proportion that are diploid (or of other ploidies) by measuring the areas under the peaks coinciding with the amount, of DNA present in the nuclei of each ploidy type. The main problem with this method is that the price of a flow-cytometer is prohibitive for most hatcheries. Nowadays, however, there are companies that have specialised in the production. : triploids of one or two of the main commercial aquaculture species and they will have their own flow-cytometers. Smaller producers can buy in readyproduced triploid seed or fry. In addition, research institutions and private companies with these facilities are not averse to providing a costed service for smaller-scale operators who may wish to carry out their own ploidy modification work.

Other methods of identifying triploids and tetraploids include direct chromosome counting, sizing of nuclei of cells from blood or other tissues and scoring genetic markers. Chromosome counting requires experience and can be quite time consuming, but has the advantage that it is a direct method. Using blood for nuclear sizing is cheap, rapid and effective and can be non-destructive but nuclear sizing of the cells of other tissues requires the sacrifice of the sampled animals. Use of genetic markers such as allozymes for ploidy confirmation relies on the fact that some polyploids will exhibit more than two alleles at highly polymorphic loci. For example, a triploid could express three alleles, with the genotype ABC. In theory, a triploid heterozygote with only two alleles (e.g. AAB) could be distinguished from a diploid (AB) by the stronger staining of the band produced by the double allele, but in practice this is not very reliable. However, the use of microsatellite loci is a great improvement on allozymes because (being highly heterozygous) they are more likely to show three . bands and (because minute amounts of DNA are required) they can be scored by non-destructive sampling.

Confirming that diploid gynogens or androgens have been successfully produced is a bit more tricky than confirming the presence of triploids

or tetraploids. Obviously, there is no difference in chromosome number, nuclear size or DNA content between normal diploids and gynogens or androgens. Here we will consider gynogens, but similar methods apply to androgens. Remember that gynogens are produced by activating eggs with sterizoa and then using a shock to suppress a cell division (meiosis I, meiosis II or first cleavage). One method that provides indirect confirmation of gynogen production is to use two controls without the shock to suppress cell division—one using untreated spermatozoa and the other using sterizoa. If everything has worked properly (sperm irradiation and the shock method) then the untreated spermatozoa control will produce normal diploid offspring while the sterizoa control will produce haploid embryos that do not survive. If these controls show the expected results then any surviving embryos from the fully treated group should be gynogens.

Sometimes the spermatozoa of a closely related species can be used to fertilise eggs, producing non-viable hybrids. In this situation sterizoa of the closely related species can be used in gynogen production. Given that hybrids are non-viable, any surviving offspring must be gynogens.

Alternatively, genetic markers can be used to confirm individuals as gynogens. For example, albinism is a recessive trait and spermatozoa from a male trout which is homozygous for pigment production (AA) can be used with eggs from an albino (aa) trout. Gynogens will all be albino (aa) and normal diploids will be pigmented (Aa). Allozyme or microsatellite loci can also be used to identify gynogens when the allele(s) present in the spermatozoa are different to the allele(s) present in the egg.

Triploids

Production of triploids is the most common target of ploidy manipulation attempts and they have a variety of uses in aquaculture. Triploids have been produced, at least experimentally, in almost all commercially aquacultured fish species, including carps, catfish, *Tilapia* and salmonids, and in most molluscan shellfish groups such as oysters, clams, scallops and abalones. One important reason for producing triploids is that they are sterile. During early gametogenesis, germ cells begin the process of meiosis and it could be that attempts to pair up the chromosomes in early meiosis are impeded by the fact that there are three homologous chromosomes rather than two. Whatever the reason, gonad development is always much reduced in triploids. Some eggs and spermatozoa *can* develop in triploids, but these are

seldom of normal ploidy and are usually aneuploid (missing chromosomes or parts of chromosomes). Triploid sterility means that, as triploid fish or shellfish reach maturity, energy that, in diploids, would go to developing gametes is available for somatic growth. As they get older, therefore, triploids effectively grow faster than diploids. Whether this potential for increased somatic growth is realised as a reduced time to market size depends on a number of factors, not least the species being cultured. Triploid fish and shellfish generally demonstrate a clear growth advantage over diploids during annual gametogenesis, but there may be more than one year of maturity before market size is reached and the advantage from the first year may not be carried over to the next.

Similarly, superior growth performance of triploids in many fish species has only been demonstrated for a small part of their life histories.

Apart from the actual or potential increase in somatic growth, there are other advantages that accrue from the sterility of triploids. Sterility makes feasible the aquaculture of non-native species or genetically depauperate hatchery-produced stock, either of which might otherwise cause adverse environmental impact if they or their gametes were to escape into the wild. Before beginning such an enterprise it is necessary to confirm, first, that all individuals are triploid and, second, that even if they produce some gametes these will never produce offspring. Considering the first requirement, methods are needed to ensure 100% triploid production and also to assess the ploidy of every individual that is to be introduced. Rather than try to perfect other triploidy induction methods, the development of tetraploids as broodstock has been the favoured option. A tetraploid individual will produce diploid gametes and these, when combined with haploid gametes from a normal diploid, produce 100% triploids. Triploids produced by this method have been called interploid triploids. Generally tetraploid males are more valuable because the diploid spermatozoa produced can be used to fertilise the eggs from a large number of females. Even though this method is quite certain to produce only triploids, regulatory bodies are wise to insist on certification that all individuals for importation are triploid, based on flow-cytometry or some other proven method, and that any gametes produced from triploid individuals are effectively non-viable.

The licensed importation of non-native, triploid grass carp into many states in the USA was only allowed when research had established