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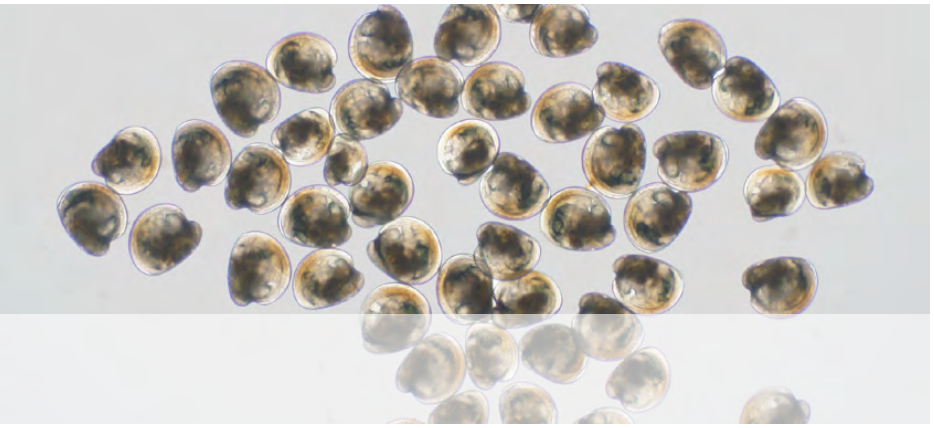
Production of triploid Pacific oyster (*Crassostrea gigas*) spat

A practical manual




Cover photographs:
Clockwise, from top left: bag system for continuous microalgae culture; cylindro-conical fiberglass larval rearing tank; triploid Pacific oyster spat; triploid oyster larvae ready to set; half-sibling diploid and triploid adult oysters grown for 18 months on the same site. ©J. Vignier/Cawthron Institute.

Production of triploid Pacific oyster (*Crassostrea gigas*) spat

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Preparation of this document

This manual represents an extensive body of research that has been carried out globally, particularly in the United States of America and France on Pacific oyster triploid seed production and aquaculture. The procedure described in the manual is largely based on the original scientific work of Standish K. Allen (Jr.) and Jon G. Stanley and their colleagues outlined in their papers Stanley *et al.* (1981), Allen (1983), and Allen *et al.* (1986) and by Andreì Geìrard, Richard Desrosiers and colleagues (Desrosiers *et al.*, 1993; Geìrard *et al.*, 1994; Geìrard *et al.*, 1999). Modifications and improvements by the authors and their colleagues have enabled this method to be implemented commercially in New Zealand.

This publication is intended as a standalone practical manual for the mass-production of triploid Pacific oysters, *Crassostrea gigas*, using a direct induction technique. It is written for hatchery staff that have extensive knowledge of breeding and growing oyster larvae and spat, and working in suitable and well equipped hatchery facilities. The authors have, however, included additional material in Appendices describing key hatchery techniques which may appear redundant for hatchery experts but be a handy guide for those less familiar with shellfish hatchery operations.

The manual starts with a brief global overview of triploid oyster production and the main benefits of growing triploid oysters. This is followed by Chapter 2 where different induction methods to produce triploid and tetraploid oysters are summarised. Methods to produce tetraploids, although very similar to the direct triploid induction method in principle, are not the focus of this manual and are only introduced and described superficially.

An extensively detailed procedure to produce large quantities of triploid Pacific oyster spat from diploids, using an optimised direct chemical induction method, is provided in Chapter 3. This step-by-step procedure includes gamete collection and preparation, controlled fertilisation, induction of triploidy in oysters, and ploidy verification of resulting larvae. Although not the focus of this manual, methodologies on broodstock conditioning, larval rearing, feeding systems, and crossbreeding tetraploid broodstock with diploid broodstock for mated triploid spat production are also presented. These techniques are only provided as a guide and may vary depending on the context of the systems used.

In Chapters 4 and 5, general considerations are outlined on the physiological performance of triploid oysters compared with diploid oysters, obtained by different induction methods. These fitness-based traits include growth, survival, reproductive potential, chromosome stability, tolerance to environmental stressors and resistance to disease. The advantages and drawbacks of each of the technical approaches to produce triploid oysters and some considerations for incorporation of triploid production into selective breeding are also summarised. Finally, a list of further readings referenced throughout the manual is provided for users who are looking for more detailed background information.

This document, produced by the Food and Agriculture Organization of the United Nations (FAO), is intended for all countries where oyster aquaculture is a growing practice. It was funded under the FAO Technical Cooperation Programme (Project: TCP/DRK/3803).

Abstract

Naturally spawned oysters are typically diploid, each cell containing two sets of chromosomes. Diploid oysters exhibit seasonal variability in condition because they spawn during the summer months following a period of ripening as the sea temperature warms. Often this coincides with the peak period for selling oysters in many countries. Triploid oysters contain three sets of chromosomes and because they are for the most part sterile, they can maintain suitable market quality throughout the year and have organoleptic qualities that are preferred by many consumers. Under the same conditions, triploids generally grow faster than diploids. For these reasons, many hatcheries produce triploid oysters.

Triploid oysters can be readily produced in hatcheries using either direct induction techniques or by first producing tetraploid oysters and then using sperm from male tetraploids to fertilise eggs from diploid females. There are several different direct techniques to induce triploidy, including physical treatments such as pressure, temperature, salinity shock or chemical treatments using either cytochalasin B (CB) or 6-dimethylaminopurine (6-DMAP).

For making tetraploids, several methods have been described but only two have resulted in the production of viable tetraploids, and only one has been successfully implemented commercially in hatcheries around the world ("indirect RUTGERS" technique). The "indirect RUTGERS" technique depends on sourcing fecund triploid females (the exception to the rule of triploid sterility) and blocking the release of the PB1, as in the IFREMER technique. The resulting cytogenetics of inhibiting PB1 in either diploid eggs or triploid eggs is profoundly different and not the subject of this manual. The "direct IFREMER" technique involves blocking the release of the first polar body (PB1) from the developing eggs of a diploid x diploid cross. Subsequently, extensive larval screening is carried out to isolate the tetraploid larvae, which are generally the smallest of the larval population.

Most hatcheries globally produce triploids by crossing tetraploids and diploids, but a method is outlined in this manual that enables direct induction of triploids using 6-DMAP with high triploid rates (typically more than 95 percent triploid) by inhibition of polar body 2 (PB2) from diploids. Broodstock must be in optimal reproductive condition to achieve high triploid induction levels and larval yields. This method is advantageous in hatcheries that carry out selective breeding because it enables the added benefits of triploidy to be integrated with genetic gains from commercial breeding programmes. In contrast, the process to integrate triploid production via tetraploids into selective breeding programmes is more complex, can be slower and is no doubt more costly.

A review of previous research comparing the performance of triploids (obtained by different production methods) and diploids in relation to growth, survival, reproductive potential, chromosome stability, tolerance to environmental stressors, and diseases reveals clear growth advantages and differences in reproductive potential in triploids. However, in some environments, survival and response to cumulative stressors and disease have been found to be more variable. Further research into these areas will add to understanding of triploid and diploid overall performance and support decision making for commercial production.

Keywords: Pacific oyster, *Crassostrea gigas*, diploid, triploid, tetraploid, hatchery, direct chemical induction, 6-DMAP.

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Abbreviations

6-DMAP	6-Dimethylaminopurine
CB	Cytochalasin-B
CUDL	Cawthron Ultra high Density Larval rearing system
DAPI	4',6-diamidino-2-phenylindole
D-LARVAE	First stage of shell formation that oyster larvae reach, shaped like the letter "D"
D-YIELD	Survival rate from the embryo stage to the D-larval stage. Also called "hatching rate"
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
FCM	Flow Cytometry
FSW	filtered seawater
GV	germinal vesicle
IFREMER	Institut Français de Recherche pour l'Exploitation de la Mer (France)
MI	meiosis I
MII	meiosis II
MSDS	Material Safety Data Sheet
MSX	*Haplosporidium nelsoni*
n	haploid
2n	diploid
3n	triploid
4n	tetraploid
OsHV-1	*Ostreid herpesvirus-1*
PB1	first polar body
PB2	second polar body
PF	post-fertilisation
PSU	practical salinity unit
SNP	single nucleotide polymorphism
USA	United States of America
UV	ultraviolet

<	less than
>	greater than
≥	greater than or equal to
≤	less than or equal to

~	approximately
µm	micron
mm	millimetre
cm	centimetre
µg	microgram
mg	milligram
µl	microlitre
ml	millilitre
L	litre
µM	micromolar
mM	millimolar
M	molar
mJ	millijoules
MΩ	million ohms
%	percent
°C	degree Celcius
Ø	diameter

1. Background

1.1 STATUS OF TRIPLOID OYSTER PRODUCTION

Triploid[1] oysters were first produced commercially in Washington State, United States of America in 1985 (Nell, 2002). Now almost forty years later, triploid oysters have become an aquaculture industry standard, accounting for around 30 percent of Pacific oyster (*Crassostrea* [*Magallana*] *gigas*) and about 50 percent of American cupped or Eastern oyster (*Crassostrea virginica*) production worldwide (Dunham, 2011). About 50 percent of Pacific oyster hatchery production in the Pacific northwest United States of America consists of triploids (Peachey and Allen, 2016), whereas nearly all commercial American cupped oyster hatchery production in the Chesapeake Bay area is triploids (Hudson, 2018). In France, hatchery production of triploid spat has been declining in recent years, with 2.15 billion spat (or ~70 percent of total hatchery production) produced in 2019. This equates to about 23 000 tonnes of market size oysters or an estimated 30 percent of the 85 000 tonnes of Pacific oysters produced in 2019 being triploids (Agreste, 2021). In New Zealand, about 50 percent of Pacific oysters harvested annually are triploids. In Australia, triploid production of Pacific oysters varies between states, ranging from 95 percent of the 190 tonnes grown in New South Wales (Wayne O'Connor, personal communication) to 10 percent of South Australia's annual 5 000 tonnes of production (PIRSA, 2023). Overall, triploid oysters represent about 15 percent of the 8 000 tonnes produced by the Australian industry. In China, 1.8 million tonnes of *C. gigas* were produced in 2022 (Li *et al.*, 2023). In Northern China, about 1.2 million tonnes of Pacific oysters were harvested in 2023, of which triploid production accounted for 80 percent (or ~1 million tonnes) of the total volume (Yuehuan Zhang, personal communication).

1.2 WHY GROW TRIPLOIDS?

Triploidy induction methods were first developed for the American cupped oyster (Stanley, Allen and Hidu, 1981) and later for Pacific oysters (Allen and Downing, 1986), and subsequently for other species (*Crassostrea sikamea*, *Crassostrea ariakensis*, *Crassostrea honkongensis*; Qin *et al.*, 2024). In most regions where oysters are consumed, the peak market for selling oysters occurs during the summer months when diploid oysters reach their reproductive maturity. These oysters become either "milky" (sexually mature) or "spent" (they spawn, and their gonadal contents [sperm and eggs] are released), which makes them unsuitable for market. To overcome this, a technique to induce triploidy was developed to prevent oysters from completing gametogenesis and reaching sexual maturity. Triploidy refers to the condition of a cell or organism having three sets of chromosomes. Such a condition in mammals is lethal, but in many species of amphibians, fish and molluscs, triploids are fully viable and do not appear morphologically different from diploids. The presence of more than two sets of chromosomes, called polyploidy, is a naturally occurring phenomenon in eukaryotes (Otto and Whitton, 2000), and triploidy can occur spontaneously at low but consistent frequencies in the Pacific oyster (Guo *et al.*, 1992).

[1] In its classification of farmed types of aquatic genetic resources, FAO classifies triploids as secondary farmed types (Mair and Lucente, 2020). Countries are reporting prevalence of triploid farmed types in Pacific oysters in AquaGRIS, FAO's global information system for aquatic genetic resources (FAO, 2024).

By inducing triploidy, gonad development and maturation cannot be fully completed. Meat yield is improved during summer months enabling the opportunity to harvest year-round even during the natural spawning season (Piferrer *et al*., 2009). A superior flesh flavour of triploids is often reported by consumers and is attributed to higher glycogen content and firmer texture (Allen and Downing, 1991; Nell, 2002). In contrast, ripe diploids have a softer texture that is generally less palatable to the consumer. This reduced reproductive effort has another side effect: in favourable conditions, triploid oysters dedicate the energy not spent for reproduction towards tissue growth and thus, grow faster than their diploid counterparts, shortening the growing time required to reach market size (Allen and Downing, 1986; Dégremont *et al*., 2012). A further benefit of triploidy, providing the induction method produces reliably 100 percent triploids, is that these oysters can be used as a method of population control to protect biodiversity by reducing the spread of introduced species (e.g. Pacific oyster) and its associated displacement of native oyster species (Guo, DeBrosse and Allen, 1996; Laugen *et al*., 2015). In some Scandinavian countries and certain estuaries in Australia (e.g. in New South Wales), farming of Pacific oysters is currently restricted by law to triploids owing to the "noxious" and "invasive" status of the species (Hansen, Dolmer and Vismann, 2023; Miller *et al*., 2014). This approach has been used effectively in crop plant breeding for many years, eliminating the risk of unplanned pollination, seed production and the undesirable spread and permanent establishment of non-native invasive species (Sattler *et al*., 2016). Another environmental benefit of using triploids is that sterility can remove the risk of genetic contamination of wild populations by selectively bred escapees (Benfey, 2016).

Although triploids offer some significant benefits compared to their diploid counterparts, there are growing concerns about the resilience of triploids and their ability to cope with stressors associated with climate change such as warming water temperatures, ocean acidification, salinity reduction and emerging aquatic diseases (see Section 4.2).

2. Induction methods for polyploidy production

Two principal approaches are utilized for the creation of triploid (3n) oysters: induced triploids and mated triploids. For the first approach, triploidy is induced by treating fertilised eggs from diploid (2n) parents with a physical shock or with a chemical solution that impairs the completion of meiosis. The success rate of physical and chemical induction methods can vary significantly, ranging from 18 to 100 percent of larvae being classed as triploid following induction (the remainder being other ploidies such as diploid, tetraploid and aneuploid); for example, see Gérard *et al.*, 1999.

The second approach to yield triploids makes use of another ploidy level, tetraploid (4n), which are individuals with four sets of chromosomes. Crossing mature tetraploid and diploid oysters results in 100 percent of offspring being triploid (Guo, DeBrosse and Allen, 1996) and the tetraploid male is always used with a diploid female.

Currently most hatcheries utilise the tetraploid approach (or "indirect Rutgers method") to produce commercial triploid oysters; however, in some places direct induction is used as it has the advantage that triploid production can be more readily integrated into selective breeding programmes.

This chapter describes the various methods of induction used to produce triploid and tetraploid shellfish, along with the reported success rates of these techniques.

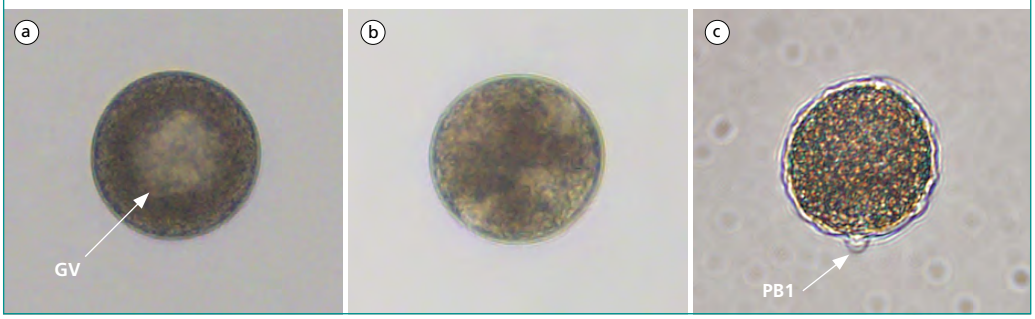
2.1 ARTIFICIAL INDUCTION FOR TRIPLOID OYSTER PRODUCTION

In mature Pacific oysters, most eggs stripped from the ovary have a germinal vesicle (GV) and are at prophase of meiosis I (Kyozuka *et al.*, 1997). Although in a small number, germinal vesicle breakdown may have occurred, and the eggs are arrested at metaphase I (Osanai, 1985). During natural spawning or upon contact with seawater following strip-spawning, meiosis proceeds until the eggs reach metaphase of meiosis I (or MI) (Figure 1). Further development is arrested until fertilisation, at which time, meiosis resumes.

In diploid oysters, fertilisation by haploid sperm (n) triggers the eggs to undergo the first round of cell division (meiosis I) and for the homologous chromosomes to separate through the release of the first polar body (PB1) (Figure 1C). Sister chromatids then separate during the second round of meiosis (meiosis II or MII) and the second polar body (PB2) is released (Figure 2, top row). The speed at which development occurs is temperature dependent.

FIGURE 1

Pacific oyster eggs (or oocytes) before (a) and after (b) germinal vesicle (GV) breakdown and after fertilisation (c) showing extrusion of the first polar body (PB1)



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The release of polar bodies during either meiosis I or meiosis II can be blocked to produce triploid embryos (Figure 2) (Guo *et al*., 1992; Gérard *et al*., 1994; Gérard *et al*., 1999; Piferrer *et al*., 2009). However, blocking at meiosis II is more commonly carried out since it results in more viable triploid larvae. There are two distinct approaches to artificially blocking polar body release to produce triploid oyster embryos: (1) physical treatments using temperature, hydrostatic pressure or salinity shocks, and (2) chemical treatments using either Cytochalasin B (CB) or 6-dimethylaminopurine (6-DMAP).

2.1.1 Physical induction treatment in shellfish

Physical treatments are generally used successfully to induce triploidy in fish but can also be applied to block the extrusion of polar bodies in various shellfish species. These include exposure of fertilised eggs (i.e. newly formed embryos) to hydrostatic pressure shock (Chaiton and Allen, 1985; Komaru and Wada, 1989; Shen *et al*., 1993), low or high temperature shocks (Barreto-Hernandez, Velasco and Winkler, 2018; Beaumont and Kelly, 1989; Osterheld *et al*., 2021; Scarpa, Toro and Wada, 1994; Toro *et al*., 1995; Yamamoto and Sugawara, 1988) or salinity shocks (Ma, Wang and Yu, 2019; Meng *et al*., 2012). The efficiencies of these induction methods – which are generally measured as triploid percentage rates and D-larval yields and indicate a method's viability for commercial application – are highly variable. For instance, the highest triploid rates reported for pressure-shocked Pacific oyster and the Akoya pearl oyster (*Pinctada fucata martensii*) embryos were 57 percent (Chaiton and Allen, 1985) and 76 percent (Shen *et al*., 1993), respectively; whereas blue mussels (*Mytilus edulis*) treated with heat-shock and cold-shock yielded triploid rates of 56 percent and 30 percent, respectively (Osterheld *et al*., 2021). Such disparities in triploid production levels are species-specific and a result of multiple factors described further in Section 2.1.2. As such, alternative methods using chemical inducers have been developed which result in higher triploid success rate and are, thus a viable alternative to commercial application in the absence of tetraploids.

2.1.2 Chemical induction treatment during Meiosis II

Chemical induction is typically achieved by treating newly fertilised eggs with either cytochalasin-B (CB) or 6-dimethylaminopurine (6-DMAP) during meiosis II (Figure 2) to stop the release of PB2; it is typically conducted between 15–30 minutes after fertilisation (Figure 2 – second row).

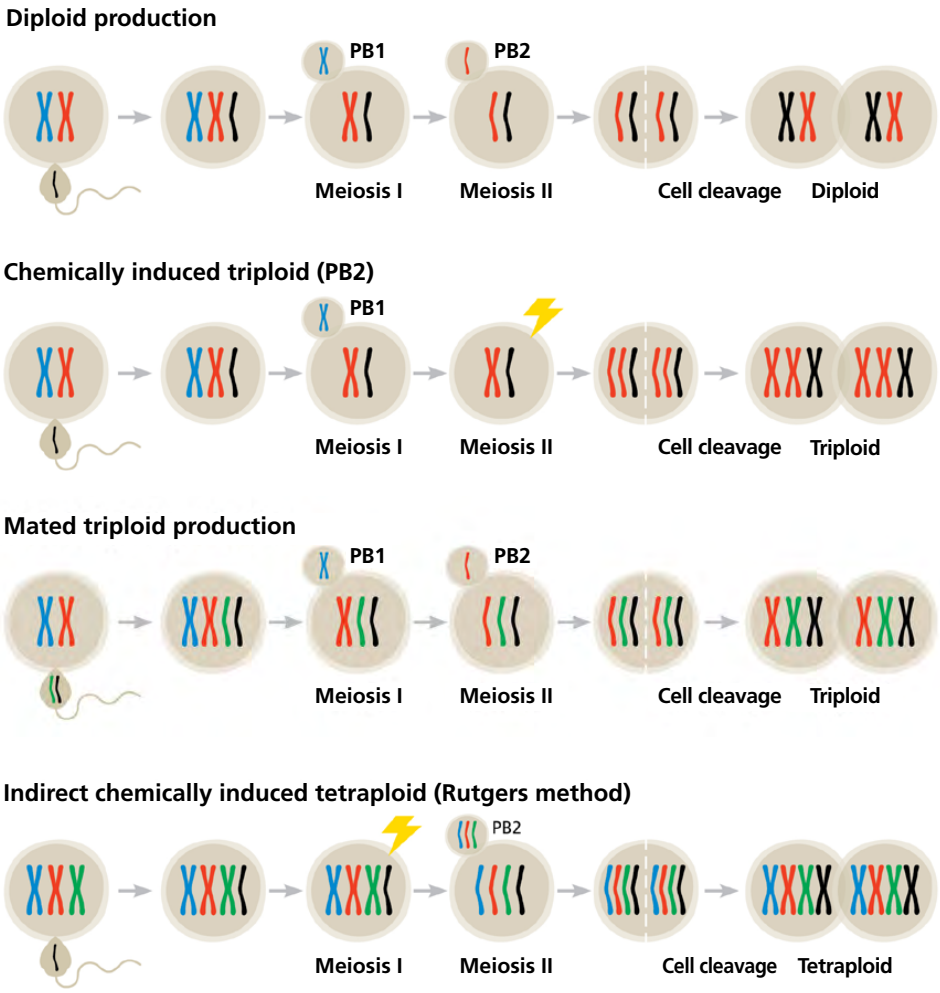
Chemical induction of triploidy can be challenging, mainly because of the lack of synchronicity in egg development. Bivalve molluscs like oysters release a considerable number of eggs during spawning which all have different degrees of maturation and are arrested at some stage prior/up to metaphase I. This differential level of maturity is especially true for eggs manually stripped out of the gonads, and failure to synchronize embryos prior to the induction treatment has often resulted in sub-optimal triploid rates (typically 40–80 percent), making the method unsuitable and inefficient for commercial use.

In addition to embryo synchronicity, there are other considerations needed to ensure successful induction and high triploid rates: seawater temperature from release to 3n induction, chemical concentration, time of initiation of chemical induction and duration of treatment (Desrosiers *et al*., 1993; Gérard *et al*., 1994).

Because the traditional chemical inducer CB and its associated solvent Dimethyl sulfoxide (DMSO) pose a severe toxicity risk to the operator, the use of 6-DMAP was investigated as an alternative to CB for inducing polyploidy in oysters. Comparison between oyster triploids and tetraploids produced by CB and by 6-DMAP resulted in similar induction success rates and comparable larval survival rates (Desrosiers *et al.*, 1993; Gérard *et al*., 1994; Gérard *et al.*, 1999; Peachey and Allen, 2016). As such, the chemical 6-DMAP is used in the induction method largely because of its enhanced safety and ease of utilisation.

FIGURE 2

Schematic representation of diploid (top row), triploid oyster production by direct chemical induction at meiosis II (second row), mated triploid production from crossing sperm from a tetraploid male with eggs from a diploid female (third row), and tetraploid oyster production via indirect (Rutgers method) chemical induction (bottom row)



In ripe females, most eggs are held in the gonad at prophase of meiosis I. Eggs mature to metaphase following release into seawater where they are halted until fertilisation resumes meiosis. Artificial induction (yellow arrow) applied during meiosis I or meiosis II can suppress cell division, producing triploids or tetraploids by retention of the first (PB1) or second (PB2) polar body. For mated triploid production, sperm (2n) from tetraploid (4n) are crossed with diploid females (1n) to obtain triploid (3n) progeny. For simplicity, in this diagram only one set of chromosomes is shown. Different colours of chromosomes represent heterozygosity.

The method of induction later described in this manual (see Chapter 3) uses 6-DMAP and can consistently yield high rates of triploid larvae as a result of improved egg preparation (thus better embryo synchronicity), and optimised factors during the induction steps (e.g. timing, temperature).

2.1.3 Determination of ploidy

The main direct techniques to determine ploidy levels in shellfish cells include: 1) measurement of DNA content via ploidy analyser/flow cytometry, 2) chromosome count via karyotyping, or 3) genotyping with DNA markers (e.g. single nucleotide polymorphisms or SNP). The widely used ploidy analyser (or flow cytometry) method requires high initial capital investment but is high throughput (i.e. numerous samples can be rapidly analysed), instantaneous, accurate, and straight forward. If

necessary, samples can also be easily fixed (typically by adding buffered formalin; see Section 3.4.3.) and analysed later. Karyotyping can be achieved at relatively low cost but is very labour intensive. Genotyping with DNA markers is costly, slow, requires specialized capabilities and is restricted to spat or adult stages.

As such, flow cytometry is the preferred method for ploidy analysis in shellfish. Using fluorescent dyes that bind to DNA molecules (such as 4',6-diamidino-2-phenylindole [DAPI] or propidium iodide), flow cytometry evaluates the cell chromosome content by detecting the intensity of the fluorescence emitted by the dye-labelled nuclei (Allen, 1983). It is therefore possible to quantify the DNA – expressed as a cumulative peak on a histogram – and establish ploidy levels using different types of cells/tissues.

2.2 TETRAPLOID OYSTER INDUCTION PROCESSES FOR SUBSEQUENT TRIPLOID OYSTER PRODUCTION

Besides inducing triploids by inhibiting polar body extrusion, an improved method, called "mated triploids", was developed in the 1990s by using tetraploid oysters (4n) and crossing them to diploids (Guo, DeBrosse and Allen, 1996). Sperm from tetraploid males (2n) is used to fertilise eggs from diploid females (1n) (see Figure 2, third row). The resulting spat are 100 percent triploid (3n). Tetraploid females are not crossed with diploid males because the progeny have been found to be abnormal, less viable and slow growing (Qin *et al*., 2023; Zhang, Qin and Yu, 2022). Furthermore, the sheer number of tetraploid sperm is orders of magnitude higher than the numbers of tetraploid eggs, making the use of tetraploid sperm more practical and efficient.

Producing tetraploids is theoretically possible by several approaches: blocking the release of PB1, gynogenesis, suppression of the first mitotic division, blastomere fusion, and using fertile triploids and performing a block at PB1 (Benabdelmouna and Ledu, 2007; Benabdelmouna and Ledu, 2015; Guo *et al*., 1992; Guo *et al*., 1993; Guo and Allen, 1994a; Stephens and Downing, 1988). In practice, many of these approaches have been unsuccessful in producing tetraploids that survive beyond spat (Guo *et al*., 1992; Guo *et al*., 1993). Several hypotheses have been proposed to explain these findings, including toxicity of the chemical inductor, expression of recessive alleles and insufficient cell cytoplasm for development (Benabdelmouna and Ledu, 2015; Guo and Allen 1994a). Two techniques have been used to produce viable tetraploids – the "indirect Rutgers" method (Patent # US5824841A; 20/10/1998) (Guo and Allen, 1998) and the "direct IFREMER" method (Patent #FR2913982-A1; 23/03/2007) – but only one ("indirect RUTGERS" technique) has been successfully implemented commercially in hatcheries around the world. The patent for the "indirect RUTGERS" method expired in January 2015, and this method to produce tetraploid oysters has been available for free commercial use since then. The "direct IFREMER" method patent will expire in March 2027.

2.2.1 Indirect Rutgers method

Viable tetraploid oysters can be obtained by inhibiting the first polar body (PB1) in eggs from fertile triploid females fertilised with haploid sperm (Guo and Allen, 1994a; Figure 2, bottom row). Obtaining eggs from triploids is possible because triploids are not 100 percent sterile (Guo and Allen, 1994b; Normand *et al*., 2009; Vignier *et al*., 2024), and some females can produce enough eggs (millions) to be used for tetraploid induction. Triploid fecundity is much less than a typical diploid female which may produce more than 100 million eggs from a single spawning event.

Eggs from fecund triploid females are collected by stripping of the gonad (i.e. incision of the gonads to collect gametes) and fertilised with sperm obtained from diploid males. At 5 minutes post-fertilisation (PF), the expulsion of PB1 is suppressed by treating the fertilised eggs with either CB (0.25–0.5 mg/L prepared in a stock solution in DMSO) or 6-DMAP (350 µM/L) for a suitable length of time (Peachey and Allen,

2016; Figure 2, bottom row). After the induction treatment, the chemical is thoroughly washed off the embryos by filtering and rinsing the treated embryos in either diluted DMSO in seawater (for CB induction) or in seawater only (for 6-DMAP induction). Subsequently, the embryos are incubated under standard hatchery conditions.

Guo and Allen (1994a) reported that many of the embryos are aneuploids (i.e. ploidy levels ranging between 2n and 3n, 3n and 4n or 4n and 5n), and only a small proportion of treated embryos were tetraploid during the first days of larval rearing. Following heavy mortalities (75 to 90 percent) of the aneuploids, tetraploid larvae became the dominant type by Day-7 PF. A typical tetraploid larval run would result in survival of larvae below 1 percent. In their publication, Guo and Allen (1994a) also showed that 3-month-old surviving spat (0.07 percent of eggs fertilised) consisted of 67 percent tetraploid, 23 percent aneuploid, 3 percent diploid, 3 percent triploid and 3 percent mosaics (oysters with a mixture of diploid and triploid cells). These yields are only indicative and should therefore be interpreted with caution. Tetraploid success varies considerably among triploid females and so it is recommended that eggs from individual females are fertilised and inducted separately (Eudeline, Allen and Guo, 2000a; Eudeline, Allen and Guo, 2000b).

2.2.2 Direct IFREMER method

The "direct IFREMER" method was established and patented in France in 2007 (Benabdelmouna and Ledu, 2007). The method produces tetraploids directly from normal diploid eggs, without any triploid parents in the tetraploid lineage (Benabdelmouna and Ledu, 2015).

Stripped eggs from diploid female oysters are fertilised with sperm from diploid male oysters at 25 °C. At 10 minutes PF, retention of PB1 is achieved by incubating the embryos with 0.5 mg/L of CB for 15 minutes. Embryos are then incubated, and larvae reared in tanks according to standard hatchery procedures with some modifications: a customised screening process designed to separate out the tetraploid larvae created during the induction process from other larvae (that maybe diploid, triploid, aneuploids or mosaics) is used.

Tetraploid larvae made by this direct induction method are typically smaller (because of slower growth) than diploid and triploid larvae reared under similar culture conditions and at the same stage of development, by 20–30 percent and 25–35 percent, respectively. Consequently, it is possible to isolate/screen different subpopulations of larvae periodically from the same batch by size into distinct size-class groups. The DNA ploidy level of each size class is then periodically verified by flow cytometry to identify the classes with the highest percentage of tetraploid larvae.

Isolation of a subpopulation rich in tetraploids and subsequent cultivation of the isolated subpopulation can be repeated several times (every 4 days), until the larvae reach the pediveliger stage (shell lengths of 220–250 µm for 4n *vs* 320–350 µm for 2n and 3n). The process can be repeated after settlement and a subpopulation of spat, typically smaller, can be isolated periodically and its ploidy status assessed. Only those size classes having the highest percentage of tetraploidy are maintained and used in subsequent triploidy production after on-growing (Benabdelmouna and Ledu, 2015).

3. Mass production of triploid oyster larvae in the hatchery via direct chemical induction

This chapter describes in depth a procedure to produce large quantities of triploid Pacific oyster spat directly from diploids using an optimised direct chemical induction method using 6-DMAP. Successful triploid production is characterised by a combined high 3n induction and hatching (survival to D-stage) yield. The efficacy of triploid production depends on numerous factors during egg preparation, fertilisation and induction processes with added influences from time and temperature. The triploid outcome is particularly sensitive to the amount of time that eggs are in seawater (and the temperature of the seawater) once they have been stripped from the gonad and before they are fertilised (time of hydration or hereafter referred to as "ageing time") as this can affect the rate that eggs undergo meiosis, developmental synchronicity and consequently, triploid success rate (see Section 3.1.2.). This factor (i.e. ageing time) along with the provision of diploid **broodstock in the best reproductive condition possible** are paramount for successful triploid production (high 3n induction success and D-larval yield). An indicative procedure for conditioning *C. gigas* broodstock is provided at the end of this manual (Appendix 1).

3.1 GAMETE COLLECTION

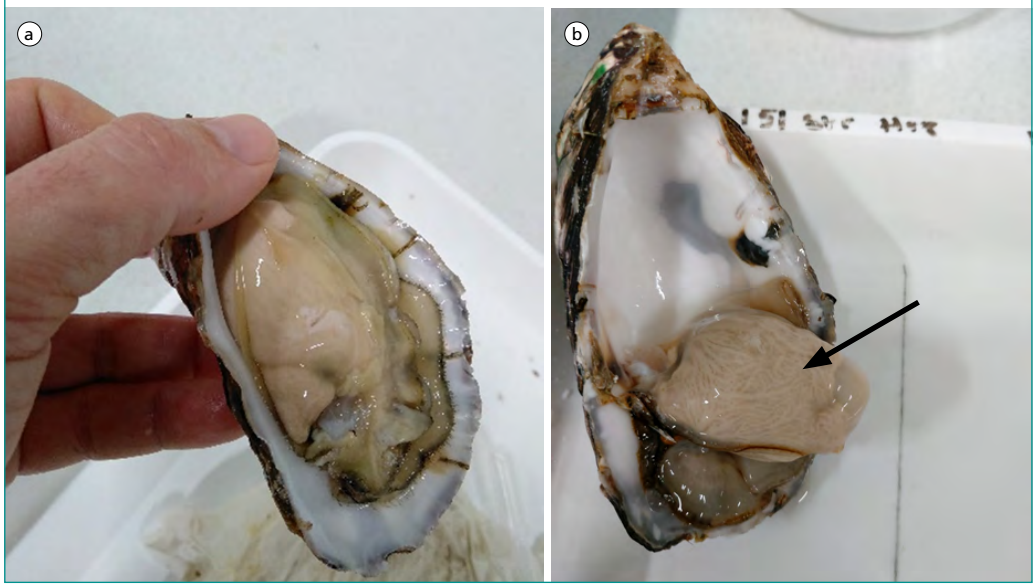
Gametes can be obtained either by physically opening broodstock and "stripping" the gonad or by inducing ripe broodstock to spawn with a change of environment (e.g. using temperature shock, desiccation, salinity changes). Typically, for both diploid and triploid production, strip spawning is used because it is efficient and allows for better control of egg development, fertilisation and avoids contamination (Allen and Bushek, 1992). There may be, however, occasions where it is desirable to retain the broodstock for later use. For example, tetraploid broodstock, because they are difficult to obtain, are very valuable from a breeding standpoint and a non-sacrificial spawning method will allow breeding across multiple generations and perpetuate valuable genetics.

For normal diploid production, eggs from several females may be combined and then fertilised but for triploid production, it is important to keep the eggs from individual females separate and then to combine larvae once the ploidy status of the larvae are confirmed.

3.1.1 Preparing for strip spawning

- Seawater should be filtered (1 µm filtered, UV treated at a dose of ≥80 mJ/cm^2), referred to hereafter as FSW, and at temperature of 25–25.5 °C for strip spawning. Note that a narrow temperature range is necessary during fertilisation and induction but thereafter, a wider temperature range (24–26 °C) may be used without any detrimental effects on subsequent larval rearing. Filtering and UV treatment maintains hygiene within the hatchery environment, reducing bacterial and pathogen loading.
- Open oysters, one at a time, and carefully remove the upper shell. The gonad of a ripe oyster will typically cover and overlie the digestive gland towards the umbone and hinge of the shell and extend around the adductor muscle (Figure 3a). A ripe gonad will have clearly visible channels ("veins") of gametes (Figure 3b).

FIGURE 3
Sexually mature or ripe diploid *C. gigas* with extended gonad (a) and a typical "veiny" appearance (b) (arrow)



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- Collect a small sample of the gonad using a clean Pasteur pipette and place it on a microscope slide.
- Assess gamete quality under the microscope (40× to 100× magnification). Good quality sperm should be vigorously motile when seawater is added; whereas viable eggs should be free of any connective tissue and have a distinct nucleus/germinal vesicle.

***Note*:** For dense gamete samples, it helps to add a small volume of clean FSW to the sample. For sperm, it will activate the motility of sperm cells and mature eggs will round up over time following dilution.

- Identify gender, label individuals accordingly, and prepare eggs and sperm as detailed below.

***Note*:** Although rare, hermaphrodites (male and female gametes both present within one individual) can be found. They should be discarded as they can be a source of uncontrolled or accidental fertilisation.

- Clean the work area well (hot water or ethanol) between each oyster to avoid contamination between individuals and to reduce the chance of accidental fertilisation of eggs.

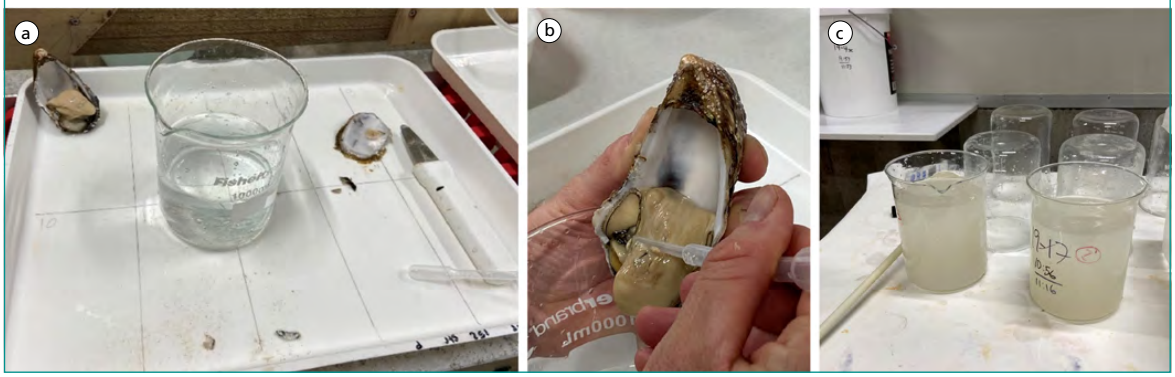
Important considerations

- Mating of related individuals should be avoided. Inbreeding is likely to impair subsequent larval and grow-out performance, especially in terms of early survival (e.g. D-yields).
- Take care not to pierce the digestive gland during stripping as this will increase the risk of contamination of the gametes with tissue, bacteria and other micro-organisms of gastro-intestinal origin which may cause issues later in larval rearing.

3.1.2 Preparation of eggs

- Strip the gonad from each oyster into FSW in a clean container (usually 1 L glass beaker). Stripping is performed by either cutting the gonad repeatedly (e.g. with a clean scalpel or a Pasteur pipette) and gently pressing and washing the exuding gametes with FSW (Figure 4).

FIGURE 4
Stripping station (a); Ripe oyster being stripped (b); Egg suspensions collected from individual females (c)



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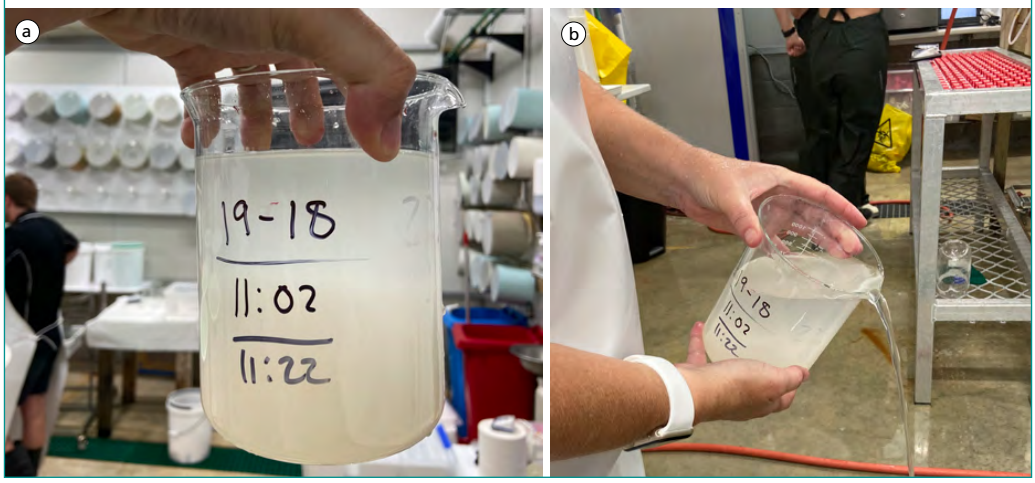
- Separate gonadal debris and tissue from the eggs by passing the egg suspension through a 55–75 µm nylon screen into a new clean beaker.

Note: Eggs will pass through the 55–75 µm screen whereas large debris will be retained on the screen.

- Store the beaker of eggs on the bench-top at stable room temperature (i.e. 25 °C). Allow eggs to settle for 15 to 20 minutes, or until a clear stratification is seen between the light poor quality/non-viable eggs and the high quality/viable eggs at the base (Figure 5).
- Once better-quality eggs have settled at the bottom of the beaker, decant the top layer, thus discarding excess seawater and poor-quality eggs (Figure 5).
- Top up the remaining egg solution with FSW at 25 °C to 900 ml for subsequent egg counts.

FIGURE 5
Stratification of the eggs can be seen 15–20 minutes after stripping with high quality eggs settled on the bottom of the beaker and light poor-quality eggs still in suspension (a). Decanting and discarding top layer of poor quality non-viable eggs (b)



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- Perform egg count for each individual female/beaker of eggs as described in Appendix 2.
- Once the 100 µl subsample has been taken from the egg suspension, gently wash eggs on a 15–20 µm screen (250 mm Ø) in clean running FSW and perform a complete drain of the container at least three times (Figure 6a).
- Carefully transfer the washed eggs from the 15–20 µm screen into a large volume of FSW (e.g. into a 20 L plastic bucket) to allow "ageing" or egg maturation to metaphase I to occur.
- Keep the egg solution at room temperature (25 °C) for an 80 to 100-minute period and maintain gentle but effective mixing of eggs approximately every 6 minutes, using a perforated plunger during this time (Figure 6b). The density of eggs should not exceed 2 500 eggs/ml (e.g. 45 million eggs in an 18 L volume of FSW).

FIGURE 6
Egg washing step, in running seawater, using a 15 µm screen (a). Egg ageing bucket with plunger (b)



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Important considerations

- Wash screens and equipment with hot freshwater followed by a rinse with FSW between use for each female.
- Do not let eggs sit in glass beakers longer than necessary. The non-viable eggs take time to sink but will settle given enough time.
- Washing eggs well removes glycogen and biological material such as gonadal debris, thus reducing the likelihood of bacterial contamination during larval rearing. Passing the eggs through a large screen (e.g. 55–75 µm) initially removes large clumps of gonad tissue. Best washing practice following decantation consists of three complete water changes and draining water from the screen holding the eggs each time between immersions.
- Repeat screening of eggs and freshly fill buckets when required to maintain stable temperature for ageing of eggs (25 °C).

- Ageing of eggs is necessary to improve ploidy rates achieved from the chemical induction as it allows eggs to undergo meiosis to metaphase I before fertilisation, ensuring that meiosis and development following fertilisation proceeds synchronously. However, extended ageing of eggs has the negative effect of decreasing D-larvae yield. It is therefore recommended to maintain ageing time between 80–100 minutes.
- Use a timer to ensure that eggs are frequently mixed with a plunger while ageing.
- Avoid cross contamination between buckets by using individual plungers or by washing plungers before use between buckets. This is especially important if making specific genetic crosses.

3.1.3 Preparation of sperm

- Strip individual males, as previously described for females in Section 3.1.2, but use only a small volume (≈10–15 ml) of FSW in a sterile sample container. Label appropriately.
- Maintain stripped sperm at 5–12 °C (e.g. in a cold cabinet until just before using for fertilisation).
- At ~15–20 minutes before fertilisation, dilute sperm 100-fold by adding 9 ml of "dry" sperm in 900 ml of FSW (25 °C) to activate it.
- Remove debris by pouring the diluted sperm through a 75–125 µm screen. Confirm sperm motility/activity under the microscope before use.

Important considerations

- Storage temperature is important, therefore access to a fridge or cold cabinet is useful with a chill bath if necessary to enable storage at the desired temperature.
- The colder the sperm has been held at, the longer the time required to resume activity.

3.2 FERTILISATION AND CHEMICAL INDUCTION

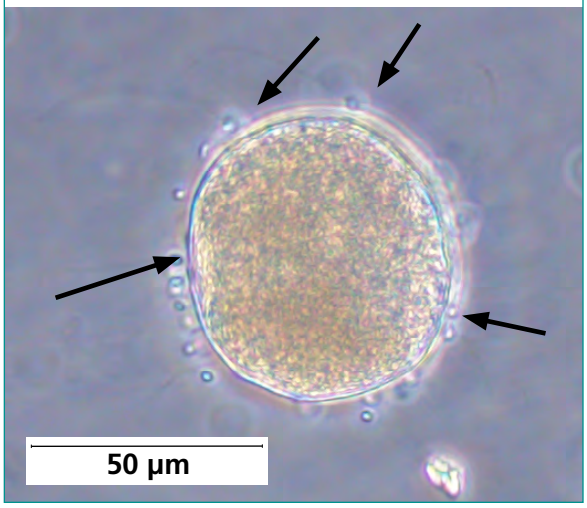
Fertilisation procedures for triploid production require more care and attention than required when producing diploid oysters. It should be carried out once the eggs have been aged long enough to allow them to reach metaphase I (80–100 minutes post-stripping). Prior to fertilising eggs, concentrated sperm is diluted and warmed to increase sperm motility and the likelihood of synchronous fertilisation being achieved amongst eggs from a given female. Typically, more sperm is added than is necessary for diploid production, also to increase the likelihood of synchronous development. While it is imperative to keep individual females separated for fertilisation and induction (i.e. single-cross) to maximize the triploid success rate, it is possible to use the same male several times for different inductions: the mating design is ultimately the decision of the hatchery managers. Once the eggs have fertilised and sufficient time has been allowed for meiosis and development to resume, the fertilised eggs are treated with 6-DMAP (450 µM Final) to block the release of PB2 during MII to induce triploidy.

3.2.1 Fertilisation procedure

- Prepare an amount of diluted sperm solution appropriate for the number of eggs to be fertilised; volume required is based on sperm to egg ratio of 1 000:1. Make up slightly more than the amount required by calculations so that more sperm can be added, if necessary.

- Drain eggs onto a 15–20 µm screen and transfer back to the same "ageing" bucket, refilled with new FSW at 25–25.5 °C.
- Adjust volume of FSW in the bucket (~5 L) to target an **egg density of 4 000–5 000 eggs/ml** at the time of fertilisation. This will allow a more effective and rapid sperm attachment to the egg. Maintain top-up water at 25 °C.
- Get timer ready for **19 minutes and 30 seconds**.
- Measure appropriate aliquot of sperm solution and have back-up available. For example, use ~200 ml of diluted sperm solution to fertilise 20 million eggs in ~5 L or 800 ml of diluted sperm to fertilise 80 million eggs in ~20 L.
- Fertilise eggs with sperm (Figure 7).
- Start the timer immediately as the eggs plus sperm are plunged to facilitate quick, gentle but effective mixing.
- Take a sample of the fertilised eggs and examine under microscope (within 20 seconds) for immediate and numerous sperm attachment to the egg surface. Ideally, 5–15 sperm should be visually observed around each egg (Figure 7) within the first minute. More sperm can be added; however, it must be within the first few minutes to ensure synchronous fertilisation and ploidy outcome.
- When satisfied with sperm:egg attachment, maintain gentle mixing every few minutes until 7–8 minutes have passed, then top-up the bucket with new FSW at 25–25.5 °C to reach 1 000–2 000 eggs/ml.

FIGURE 7
Oyster egg photographed one minute after fertilisation. Note sperm attachment (black arrows) around the egg (here ~12 spermatozoa)



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Important considerations

- Temperature affects the speed of biological processes. Therefore, **maintain temperature at range of 25–25.5 °C for fertilisation and induction processes**. If the water temperature is outside target range of 25.0–25.5 °C **STOP** and correct before proceeding with fertilisation stage.
- Ensure that sperm solution is retrieved from the cold cabinet for at least ~10 minutes before use and warmed up to ambient for optimal fertilisation outcome.
- Keep fertilised eggs suspended (off the container's bottom) and assist movement of water periodically throughout fertilisation development stage.

3.2.2 Chemical induction method

The release of the second polar body in fertilised eggs is blocked with 6-DMAP (450 µM Final). For best results, a stock solution of 6-DMAP is made up daily. Operators should be familiar with the Material Safety Data Sheet (MSDS) for this chemical before using.

Although the chemical is of low toxicity (see Appendix 5), gloves and eye protection should be worn when handling both stock and working solutions of the chemical (see Appendix 3).

- At 18 minutes following fertilisation, start to drain the fertilised eggs on a clean 15–20 µm (250 mm Ø) screen submerged (e.g. use of an 8 L square container as shown in Figure 6a) in running 25 °C seawater. This will wash off the sperm

from the fertilised eggs and improve the subsequent chemical induction. Have fertilised eggs well drained in time to transfer to the prepared 6-DMAP (450 µM) treatment bath at **19 minutes 30 seconds**, post addition of sperm.

- When the timer rings (end of the 19 minutes 30 seconds post-fertilisation period), immediately immerse fertilised eggs on the 15 µm screen in the 6-DMAP treatment solution (Figure 8). Start the time for **10 minutes**.
- During the 10 minutes induction period, **agitate gently and constantly**. Ensure effective mixing of the 6-DMAP treatment solution around the biomass of eggs.
- Twenty seconds before treatment time is up, move eggs on screen to avoid clogging in anticipation of removal from the treatment bath.

Note: If a large number of eggs is being treated (35–50 million), it will be hard to drain the 6-DMAP solution completely, therefore, move gently the eggs to avoid clogging the sieve while in 6-DMAP treatment bath, especially at the end of treatment time.

FIGURE 8
Chemical treatment of fertilised oyster eggs with 6-DMAP in seawater to induce triploidy



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- At 10 minutes exactly, transfer the eggs from the treatment bath to a new FSW rinse bath to wash off the 6-DMAP solution. Repeat in new rinse bath of FSW.
- Transfer the treated eggs to an incubation tank by delicately rinsing the back of the 15–20 µm screen using FSW.
- Stock the incubation tank at **100–150 eggs/ml** (typically a maximum of 25 million treated eggs are stocked in a 150 L incubation tank).

Important considerations

- Timing is important. Chemical treatment should start at precisely 19 minutes 30 seconds to prevent the extrusion of PB2.
- Avoid cross contamination and subsequent effect on ploidy rates by thoroughly washing all equipment, hands, aprons with hot freshwater between inductions.
- Good drainage and rinsing are necessary to remove residual traces of chemical. Delay at this point could influence 3n success rate and D-yield.
- It is possible to re-use the 6-DMAP working solution.

3.2.3 Mated triploid method

As previously described, the production of mated triploids is the result of crossbreeding a tetraploid male with a diploid female. The predictability and efficiency of the mated triploid technique to produce large volumes of triploid spat has led to its adoption around the world as it essentially follows the same process used to crossbreed between diploids. For this section, it is assumed that ripe tetraploid (4n) broodstock are readily available. Details for producing tetraploid broodstock are outlined in Section 2.2.

- Collect sperm from 4n males by either stripping (sacrificial; see Figure 4b and Section 3.1.3) or by inducing them to spawn by thermal cycling as described in Helm, Bourne and Lovatelli (2004).

- Check cell motility under the microscope and store the concentrated sperm between 5 and 12 °C.
- Confirm the ploidy status of the male parent by collecting:
  - a piece of gill (if sacrificial strip-spawning is used) and process for ploidy analysis as described in Section 3.4.2, or
  - a subsample of sperm and process for ploidy analysis as described in Section 3.4.3.

- To prepare the sperm for fertilisation, follow the same procedure as described in Section 3.1.3.
- Collect eggs from diploid females by strip-spawning. For preparation of the diploid eggs, follow the same procedure as previously described in Section 3.1.2.
- Once eggs are washed and counted, transfer them into FSW for 20–40 minutes for ageing or hydration.
- Fertilise the eggs following the same procedure as described in Section 3.2.1. Due to the slower 4n sperm velocity, double the volume of sperm to target a sperm to egg ratio of 2 000:1. Ensure that numerous sperm cells are attached to the egg surface with the first few minutes (5–15 sperms/egg ratio is targeted).
- After at least 15 minutes post-fertilisation, wash off excess sperm by screening the fertilised eggs on a 15–20 µm sieve. Transfer the fertilised eggs to the incubation tank.

3.3 EMBRYO INCUBATION

Once eggs have been fertilised and triploidy has been induced, eggs are then transferred to incubation tanks where they are left for 20 hours or more, depending on the temperature, to develop to the D-larval stage. This process is much the same as it is to produce diploid oysters and at the end of this period, oysters can be tested to confirm triploidy rate and D-larval yield before being pooled for subsequent larval rearing.

- The day before spawning/induction, prepare the incubation tanks – cylindro-conical or large flat-bottom tanks are generally used – by filling with FSW (i.e. 1 µm filtered, UV-treated seawater) at a temperature of 25–26 °C (±1). Depending on the quality of the source seawater, pH can be adjusted to 8.3–8.4 using sodium carbonate and Ethylenediaminetetraacetic acid (EDTA) (4 to 12 µM) can be used to chelate toxic compounds such as heavy metals. In general, incubation water is aged for a minimum of 20 hours with vigorous central aeration to ensure optimum EDTA chelation.
- Reduce aeration prior to embryo addition to small intermittent bubbles so as not to damage the delicate embryo stages.
- Stocking density in incubation tanks should not exceed 150 embryos per ml for optimal embryo-larval development and high D-yields.
- Operate tanks in static for the duration of incubation (i.e. no water exchange).
- Seawater in prepared tanks will equilibrate to the surrounding air temperature. Climate control in the rearing area will assist to maintain optimal seawater temperature at this time (generally 25 °C). A wider temperature range can be tolerated by the embryos (20–30 °C). However, this will affect the speed of embryonic development and/or normal development into D-larvae. A temperature close to what will be used to rear the larvae should be targeted and maintained.
- Only pool individual inductions at the end of the D-larval incubation once the triploidy rate of a particular male/female cross and induction is known.
- Ploidy status is generally determined at D-larval stage when abnormal embryos can be readily screened out and separated. See Section 3.4.1.

3.4 PLOIDY LEVEL DETERMINATION FOR ALL STAGES

Several methods can be used to determine ploidy status (see Section 2.1.3). Ideally this is done at D-larval stage so that labour effort is not wasted if induction has not been successful.

DAPI or 4',6-diamidino-2-phenylindole is typically used to stain the DNA of cell nuclei prior to analyses with the ploidy analyser/flow cytometer. Triploid nuclei will have a peak that is 1.5× that of diploid cells. Cells undergoing division may also be detected (i.e. with 2× the fluorescence of diploid and triploid nuclei, respectively). There are several stain recipes published in the literature using DAPI; a stain recipe is provided in Appendix 4. Most hatcheries use the proprietary reagent Cystain® UV Ploidy solution (Sysmex, Sysmex Flow Cytometry, USA). Note that the CyStain® reagent is photosensitive. Therefore samples, once stained, should be maintained in the dark to ensure triploids and diploids can be readily differentiated if there is any delay in analysis.

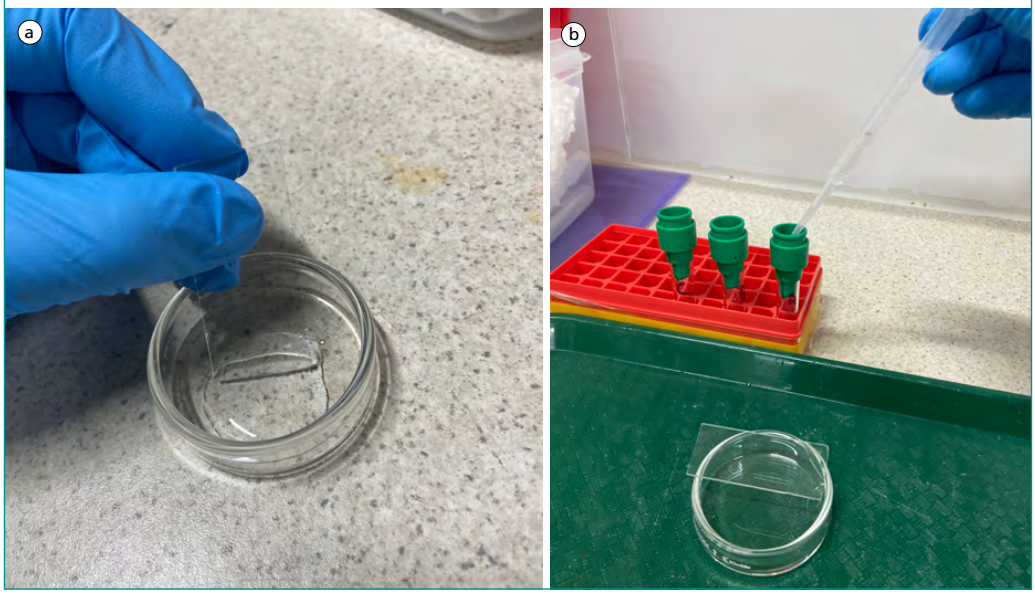
3.4.1 Ploidy determination of D-larvae

Typically, Pacific oyster larvae reach the swimming D-larval shelled veliger stage (mean shell length of 70±2 µm) at approximately 20 hours post-fertilisation at 25 °C (±1). Below is a procedure modified from Allen and Bushek (1992) that is used routinely to determine the percentage of triploids from a given larval sample/tank.

- Ensure a fresh sample of diploid (2n) larvae is available to be analysed concurrently for calibration of ploidy peaks. To do so, a diploid control (not subjected to 6-DMAP induction) should be made on induction day. Alternatively, a sample of larvae previously frozen at -20 °C could be used.
- On the day following induction, collect a subsample of larvae from the top of each incubation tank to obtain ≥10 000 larvae (e.g. ~1 000 ml for a tank stocked with 100 eggs/ml).
- Pour the subsample onto a 43 µm screen to collect the larvae and wash gently with cold freshwater to separate the larvae from the abnormal and not-developed embryos.
- Concentrate the larvae into approximately 1.5 ml in suspension with cold freshwater. The use of freshwater will help disaggregate larval tissue.
- Collect with a clean Pasteur pipette and place in a glass Petri dish. Larvae should be visible on the glass surface.
- Crush the larvae using a glass slide to macerate the larvae against the Petri dish for a homogeneous mixture of cells (Figure 9a). Each sample should contain enough DNA to give meaningful ploidy results (generally >10 000 events/cells are processed).
- Add 1.5 ml of DNA stain solution (DAPI/Cystain® Ploidy solutions) to the homogenised larvae (Appendix 4).
- Pass the cell suspension through a sterile CellTrics® 30 µm filter (Sysmex, Sysmex Flow Cytometry, USA), into an appropriate sample test tube (Figure 9b).
- Run the sample immediately using a Ploidy analyser. For example, CyFlow™ Ploidy Analyser (Sysmex, Sysmex Flow Cytometry, USA) (Figure 10a), Partec™ Ploidy Analyser or similar.
- If the sample is too concentrated, this will result in too many events (i.e. single particles/cells detected at the same time by the instrument) and potentially also incorrect readings. If this occurs, dilute the sample further by adding more DAPI stain solution or equivalent through the CellTrics® filter.
- Calculate the proportion of cells in each ploidy class relative to the proportion of observations in all ploidy classes. Typically, each ploidy peak is highlighted, and the ploidy analyser automatically calculates the number of events/cells that occur

FIGURE 9

A sample of putative triploid oysters is homogenised for subsequent ploidy analysis by crushing larvae using a glass slide (a); Cystain® Ploidy solution (or a DAPI solution) is added to the homogenised larvae which have previously been filtered through the CellTrics filter (green) into the test tube (b)



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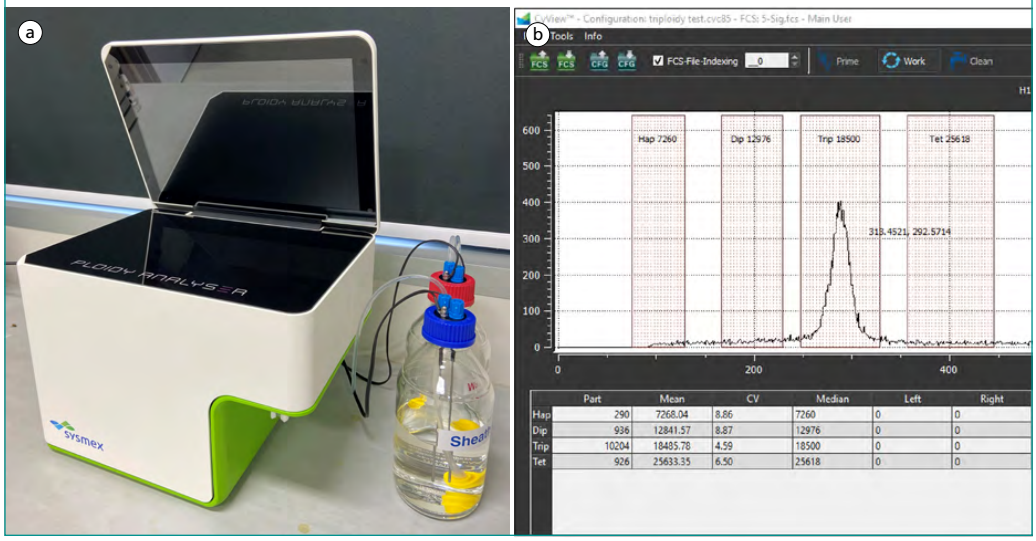
in each peak (Figure 10b). The percentage of larvae estimated to be triploid is the percentage of cells represented by the triploid peak and the triploid "doublet" peak (cells that have 2× the fluorescence of triploids as they are undergoing mitosis), divided by all the cells that have been counted (diploid, diploid doublet cells, etc.).

- Once results are obtained, select the successful induction tanks (typically, 3n proportion ≥95 percent), drain and collect the D-larvae from each tank for pooling and distribution to the larval rearing tanks (see Section 3.5.1). Any tanks that have a low 3n yield (typically <95 percent) can be discarded.

FIGURE 10

Ploidy analyser (CyFlow™ Ploidy Analyser, Sysmex) that measures relative DNA content in cells (a). Example of triploid peak (100% triploid) obtained on a CyFlow™ Ploidy Analyser (b)



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3.4.2 Ploidy determination of adults using tissue samples

- Open the oyster.
- Collect a square piece (2 × 2 mm) of tissue from the base of the gill.
- Use tweezers and scissors.
- Rinse the tissue sample with clean FSW.
- Place the tissue sample on a glass Petri dish.
- Using a glass slide, macerate the tissue against the Petri dish. Alternatively, disaggregate the tissue sample in an Eppendorf tube using a pipette or by repeating aspiration with a 1 ml syringe fitted with a 26G needle.
- Add 1.5 ml of DAPI/Cystain® ploidy solution (see Appendix 4).
- After 1 minute, pass the cell suspension through a sterile CellTrics® 30 µm filter (Sysmex, Sysmex Flow Cytometry, USA) into an appropriate tube (Figure 9b).
  Note: Alternatively, place the macerated tissue sample directly in the DAPI/Cystain® Ploidy solution, and then filter before running the sample on the ploidy analyser.
- Run the sample immediately using a ploidy analyser (e.g. CyFlow™ Ploidy Analyser, Partec Ploidy Analyser or similar).
- Ensure a fresh sample of diploid (2n) tissue is available to be analysed concurrently for calibration of ploidy peaks. Cells of a triploid oyster will have a peak that is 1.5× the fluorescence of a diploid oyster. Similarly, cells of a tetraploid oyster will have a peak that is 2× the fluorescence of a diploid oyster.

3.4.3 Ploidy determination of adults using sperm

- Collect 0.1–0.2 ml of naturally released sperm (or dilute sperm 100-fold if collected by stripping the gonad from the adult).
- Pass the sperm suspension through a sterile CellTrics® 30 µm filter (Sysmex, Sysmex Flow Cytometry, USA) into an appropriate tube.
- (Optional) Add 0.5 ml of buffered 10 percent formalin and store at 5 °C for later analysis.
- Add 1.5 ml of DAPI/Cystain® ploidy solution (see Appendix 4).
  Note: Alternatively put the sperm sample directly in the DAPI/Cystain® Ploidy solution, and then filter before running the sample on the ploidy analyser.
- Run the sample immediately using a ploidy analyser (e.g. CyFlow™ Ploidy Analyser, Partec Ploidy Analyser or similar).
- Ensure a fresh sperm sample from a diploid male is available to be analysed concurrently for calibration of ploidy peaks.
- The ploidy peak for a tetraploid male will be at the same fluorescence as 2n cells, whereas the ploidy peak for diploid sperm cells should be at the equivalent of 1n (half the fluorescence of the diploid cells).

3.5 LARVAL REARING PROCEDURE

For artificially induced triploids, the larval rearing procedure generally follows that of standard diploid larval rearing which has been extensively described by Helm, Bourne and Lovatelli (2004) and more recently by Sarkis and Lovatelli (2022), with some additional steps. Specifics of rearing triploid larvae are provided in Section 3.5.2.

Larval rearing starts from day-1 PF when D-larvae of known ploidy status are recovered from the incubation tanks and stocked in larval rearing tanks. The rate of larval development can vary and is primarily dependent on the seawater temperature used for rearing, but also can be influenced by genetics (selected vs wild genotypes) and ploidy level. Pelagic/swimming larvae are reared in clean sterile seawater and fed hatchery-grown microalgal feed to provide optimal daily growth until settlement is reached, and larvae become benthic. Generally, it takes about three weeks at 23–26 °C for larvae to develop to the eyed stage which indicates that they are ready to settle. Larvae are then set and reared as spat for the subsequent nursery phase. Larvae can be

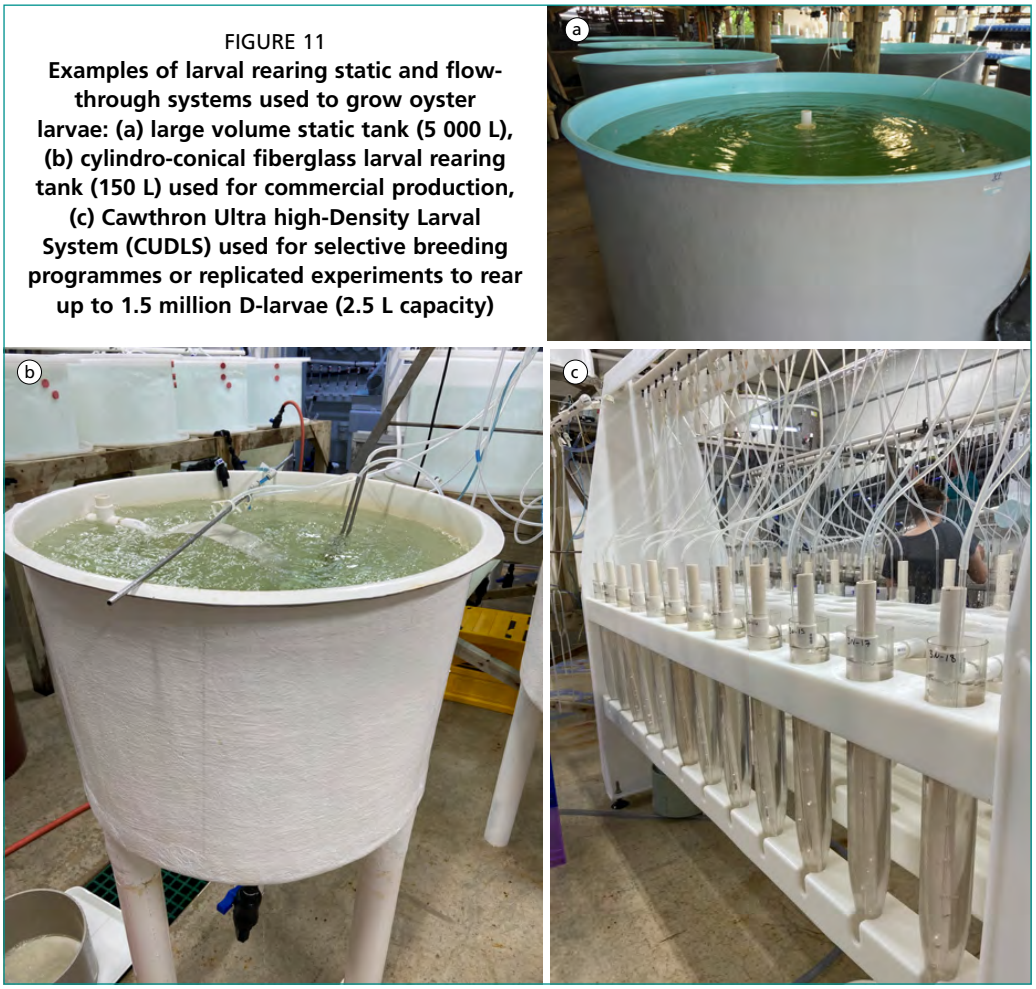


FIGURE 11
Examples of larval rearing static and flow-through systems used to grow oyster larvae: (a) large volume static tank (5 000 L), (b) cylindro-conical fiberglass larval rearing tank (150 L) used for commercial production, (c) Cawthron Ultra high-Density Larval System (CUDLS) used for selective breeding programmes or replicated experiments to rear up to 1.5 million D-larvae (2.5 L capacity)

reared in either static or flow-through systems, or a combination of both. Typically, static or batch rearing systems consist of large volume tanks (200–50 000 L; Figure 11a) stocked at relatively low larval densities (<10/ml). They require complete water exchanges every 2 to 3 days to maintain "optimal" seawater culture conditions (Sarkis and Lovatelli, 2022). Flow-through rearing systems are much smaller in volume and hatchery footprint (2–200 L; Figure 11b & 11c), and can grow larvae at very high densities, provided that a continuous flow of clean seawater and sufficient microalgal food are supplied.

3.5.1 Recovering D-larvae

This is the first step of larval rearing. It is therefore important to ensure the best quality larvae are recovered from the incubation tanks. Hygiene and health of the larval population is supported by efficiently screening out all the abnormal embryos and undeveloped and non-viable larvae. This is also when the starting quantity of larvae for rearing can be estimated accurately.

- Drain each incubation tank through a 43 µm screen. To prevent possible losses of larvae caused by holes or tears in primary screens, place a secondary backup 43 µm screen underneath the primary screen to catch possible escapees.
- Wash D-larvae retained on the 43 µm screen delicately with FSW. Move the larvae around the screen while washing to help separate the abnormal embryos and non-viable larvae (which will go through the screen) from the viable larvae.
- Collect the viable larvae in a clean container (1 to 5 L) and proceed to counting of larvae for estimation of survival to the D-larval stage (i.e. D-yield).

- Follow counting procedure detailed in Appendix 2 with minor changes:
  - Use a 1:10 dilution step, if required.
  - Collect 3 aliquots of 25–50 µl.
  - Add one drop of Lugol to fix larvae.
  - Proceed to counting.
- Once the number of D-larvae are known, transfer the larvae to the larval rearing vessels at the following stocking densities:
  - If batch larval rearing is conducted (i.e. semi-static renewal system where water is changed every 2 to 3 days), aim to stock initially between 10 and 20 larvae/ml of rearing seawater.
  - If flow-through larval rearing is conducted (i.e. complete water exchange every 1–2 hours), aim to stock between 50 and 500 larvae/ml of rearing seawater.

3.5.2 Rearing triploid larvae

The larval rearing procedure used for artificially induced triploids generally follows that of standard diploid larval rearing with some additional steps. Batches of triploid larvae will typically still have aneuploid/abnormal larvae amongst the population during the first week (at a much higher incidence than occurs in diploid production). Triploid larvae also have faster growth rates and are a larger size at competency/settlement (Table 1). Here are some recommendations for effective triploid larval rearing:

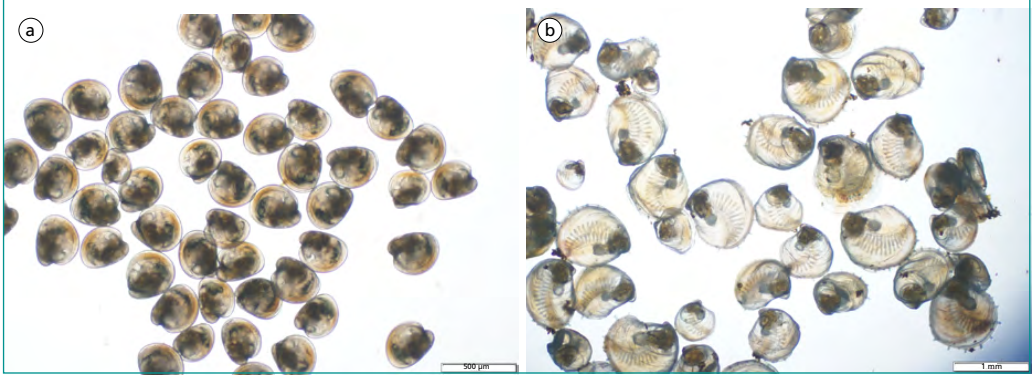
TABLE 1
Comparative mean shell growth (± Standard errors, SE) of chemically induced triploid *Crassostrea gigas* larvae with their diploid counterparts grown at 23 °C (±1). A total of 150 larvae were measured for each ploidy group, at each timepoint

Age larvae (post-fertilisation)	Mean shell lengths in µm	
	Diploid	Triploid
Day 1	73.0 (0.5)	74.8 (0.6)
Day 3	81.5 (0.1)	84.6 (0.1)
Day 6	104.3 (0.1)	107.7 (0.1)
Day 8	134.9 (0.2)	133.2 (0.2)
Day 12	233.3 (0.1)	237.5 (0.4)
Day 15	307.2 (0.3)	314.8 (0.6)
Day 23	540 (1.2)	580.6 (2.2)

- Cylindro-conical fiberglass tanks equipped with a bottom drain are typically used (Figure 11b); capacities/volumes range from 150 L to up to 20 000 L (Figure 11a) based on the larval rearing method chosen (batch vs flow-through). Larval rearing tanks are aerated from the central base of the tank.
- Seawater should be mechanically filtered to at least 1 µm, and UV-sterilized (80 mJ/cm^2) to kill any pathogens and unwanted micro-organisms; additional treatment with activated carbon filters is recommended if there is concern of residual chlorine by-products or other dissolved organics or metals. Optimal seawater temperature and salinity should be maintained between 22 and 28 °C and from 25 to 35 PSU, respectively.
- Procedures for rearing triploid oyster larvae follow methods previously described for diploids (Helm, Bourne and Lovatelli, 2004; Sarkis and Lovatelli, 2022), with minor adjustments. Specific points of differences to consider are:
  - Grading is performed periodically (every 1 to 3 days) using an appropriate screen size to remove/cull the abnormal larvae and slower growing animals:
    - → During week-1, **10 to 20 percent** is culled per grading.
    - → From week-2 onwards, **5 to 10 percent** of the population should be culled at

each grading to support healthy growth of the batch. Triploids grow faster and are generally larger than their diploid counterparts (Table 1).
    - Strict adherence to periodic screening/culling as well as washing will assist in maintaining healthy rearing conditions in the larval tanks and mitigate the risks of bacterial contamination and crashes.
- Larval rearing systems require daily maintenance. In addition, health assessment of the stocks – including larval mortality, swimming activity, tank hygiene (absence of bacteria and ciliates), larval feeding, residual algae, larval colour, growth (shell length) – should be conducted daily.
- Microalgal food cell concentrations need to be maintained at levels conducive to efficient feeding activity. For more details on microalgal culture and larval feeding strategy/regime, refer to Helm, Bourne and Lovatelli (2004) and/or Sarkis and Lovatelli (2022).
- When triploid larvae reach competency (i.e. when an eye spot is present in >80 percent of the larval population, when larvae exhibit crawling behaviour and/or are producing mucus) (Figure 12a), collect larvae on a screen (236 to 250 µm) to prepare for settlement. Generally, triploid larvae will reach settlement a day earlier than their diploid counterparts reared under the same conditions.

FIGURE 12
Competent triploid larvae at the eyed-stage (a); newly metamorphosed single-seed spat at 4-days following epinephrine treatment (b)



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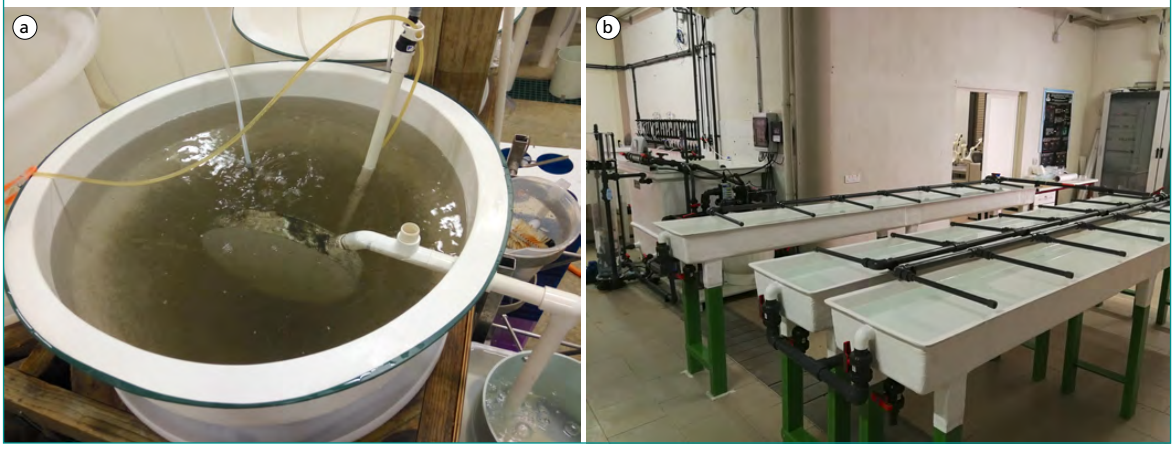
- Transfer to settlement unit for production of single-seed oysters (Figure 12b). Settlement is carried out using two techniques:
    - Chemical induction of settlement using epinephrine. Eyed larvae (>236 to 250 µm screen size) are treated with 10^{-4} M epinephrine for 1 hour in a conical tank (Figure 13a) with strong central aeration. New FSW is then added, in flow-through, and the epinephrine is flushed out. Single seed spat are collected the following day on a 275 µm screen and on grown.
    - Settlement is carried out using micro-cultch (or crushed oyster shells of 200–300 µm size range) in shallow raceway-type tanks equipped with downwelling flows (Figure 13b). Eyed larvae (previously screened on 236/250 µm) are added to micro-cultch and will attach to single shell particle. Stocking rates are generally around 100 000 larvae for 60 g of micro-cultch. Newly settled spat are recovered after several days using a 300 µm screen.

Once young triploid spat are recovered from the settlement units, they can be transferred to nursery units where they are then reared (e.g. upwelling systems) until they are deployed at sea for further grow-out, as per standard diploid culture techniques (Helm, Bourne and Lovatelli, 2004; Mercer, Gennari and Lovatelli, 2024).

FIGURE 13

Conical settlement tank equipped with a banjo filter and stocked with epinephrine-treated oyster larvae (a). Raceways used for downwelling settlement on micro-cultch (b)



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4. Hatchery and field performance of triploid oysters

4.1 COMPARATIVE HATCHERY PERFORMANCES OF 3n AND 2n LARVAE

There are limited data available on the early larval performances of triploid oysters compared with diploids and how physiological requirements may differ between ploidy types (Brianik and Allam, 2023). This knowledge gap is largely because of (i) the variable yields of triploid induction success associated with triploid induction methods, and (ii) the lack of equivalent genetic background when conducting an experiment to assess the influence of ploidy on performance.

Triploid larvae (produced by blocking of PB2 using 6-DMAP) have been found to have higher mortality rates and lower D-yields than the rates of their diploid sibling equivalents (Vignier *et al*., 2024). Similarly, directly induced triploid oysters (meiosis I and II) have both been found to exhibit higher mortality rates than diploid controls, particularly during early embryo and larval development (Desrosiers *et al*., 1993; Gérard *et al*., 1994; Gérard *et al*., 1999) (Table 2). This observation was observed also in later larval development (from D-stage to early spat stage), with artificially induced (6-DMAP) triploids experiencing lower survival compared with their full sibling diploids (Vignier, 2017; Vignier *et al*., 2024). Comparative studies of larval performances of triploids produced from CB blocking at MI and MII found that batches of MI-induced triploids often experience higher mortality than batches of MII triploids due to the higher proportion of aneuploid larvae amongst the population (Stanley, Hidu and Allen, 1984; Gérard *et al*., 1999) (Table 2).

Reliability and efficiency of triploid production has been greatly improved by the development of the "mated triploid" method. Guo, DeBrosse and Allen (1996) and more recently Vignier *et al.* (2024) noted that triploid larval production of Pacific oysters from mating tetraploids with diploids was as simple as producing normal diploid larvae, with comparable survivorship to spat stages (Table 2). Improved survival observed in mated triploids can be explained by the reduced numbers of non-viable aneuploids, typically caused by meiosis II-chemical induction. For this reason, crossbreeding tetraploids with diploids has become the industry standard to produce triploid Pacific oysters, worldwide.

Growth comparison of artificially induced triploids (PB2 blocking by 6-DMAP) and their full-siblings diploids larvae have revealed a significant advantage of triploids from hatching (D-larvae) to beyond spat stage (Vignier, 2017; Vignier *et al*., 2024) (Tables 1 and 2). Triploid gigantism was proposed as one possible cause to explain superior growth prior to sexual reproduction (Guo and Allen, 1994c).

4.2 COMPARATIVE FIELD PERFORMANCES OF 3n AND 2n OYSTERS

For the reasons previously mentioned, there is limited information available that compares the performance of triploids produced from artificial inhibition of MII with those of mated triploids directly, as genotype-environment interactions can significantly interfere with estimates of ploidy performances.

4.2.1 Growth

Allen and Downing (1986) were some of the first to quantify the performances of triploid Pacific oysters relative to their diploid counterparts. The enhanced performance in triploids, referred to as the "triploid advantage" by Callam, Allen and

FIGURE 14
Comparison of half-sibling diploid (a) and mated triploid (b) Pacific oyster after 18 months of grow-out on the same farm site



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Frank-Lawale (2016), can be hypothesized to be the result of physiological changes due to partial sterility and to energy reallocation (Figure 14). Energy reallocation is apparent once oysters reach sexual maturation, where triploids will reallocate time and energy from gametogenesis to somatic growth (Allen and Downing, 1986; Guo, DeBrosse and Allen, 1996).

Increased heterozygosity has been suggested to explain growth advantage typically observed in triploid oysters. Chemically induced triploids are made of two sets of chromosomes derived from a female diploid, and the two chromosomes are either genetically different (retaining the first polar body during meiosis I) or identical (retaining the second polar body during meiosis II). This genetic distinction results in meiosis I-induced triploids being more heterozygous (genetically diverse) than meiosis II-induced triploids. Wang *et al.* (2002) confirmed this hypothesis and determined a strong correlation between oyster meat weight at 1 year of age and heterozygosity. Triploid Pacific oysters (*C. gigas*) produced from diploid × tetraploids mating were 10 percent bigger than triploids produced from meiosis II inhibition with CB (Wang *et al*., 2002) (Table 2).

The triploid advantage was clearly established by Dégremont *et al*. (2012) who compared the growth of selected mated triploid American cupped oysters with selected diploids and found that triploid oysters were 88 percent heavier and 25 percent larger and had a significantly higher meat yield (+152 percent) than diploid cultured oysters from the same sites (Table 2). However, environmental conditions such as low salinity or elevated temperature can greatly affect the performance of triploids, which may respond differently than diploids. Callam, Allen and Frank-Lawale (2016) found that under low salinity conditions (6–13 PSU) triploid American cupped oysters had similar growth to diploids. On the other hand, several studies reported that under unfavourable conditions (multiple co-occurring stressors such as low salinity, high disease pressure, and poor food availability), triploids grew faster than diploids (Garnier-Géré *et al*., 2002; Goulletquer *et al*., 1996). In terms of temperature, Ibarra *et al*. (2017) found

that at temperate sites triploid Pacific oysters had 18 percent greater weight gain than diploids, whereas at tropical sites the outperformance was more marked with triploid oysters gaining up to 70 percent more weight/month (Table 2).

4.2.2 Field survival rates

Inconsistent results have been found when comparing the survival of triploid with diploid oysters out on the farms. Some studies have reported greater survivorship among triploids (Dégremont, Soletchnik and Boudry, 2010; Matthiessen and Davis, 1992; Pernet *et al*., 2012), whereas others have recently reported triploid-specific mass mortality events, referred to as "triploid mortality", in both Pacific oysters and the American cupped oyster (Guévélou *et al*., 2019; Houssin et al., 2019; Matt *et al*., 2020; Wadsworth *et al*., 2019). To date, these mass mortality events have not been associated with any known pathogens, although the induction method (mated triploid vs chemically induced triploid) appears to be an important factor when comparing survivorship, particularly amongst most recent reports (Brianik and Allam, 2023). Recent studies have also highlighted the synergistic effects of multiple co-occurring stressors (e.g. low salinity, hypoxia, heatwaves) on oysters' physiological processes leading to higher mortality risks in triploids (Casas *et al*., 2023; George *et al*., 2023).

High summer temperatures typically coincide with sexual maturation of oysters, suggesting that because oysters dedicate more energy towards the production of gametes, there is less energy available for immune defence. Due to their suppressed gametogenesis, triploid oysters are thus expected to experience lower mortality than diploids during summer. However, this is not always what has been observed. In a comparative study by Goulletquer *et al*. (1996), triploid Pacific oysters were produced by treatment of the eggs (PB2) using CB and deployed in the field. Compared to the diploid controls, triploids showed significantly lower survival after 26 months, i.e. 68.9 percent for 2n and 48.6 percent for 3n, respectively. When investigating the summer mortality events in France, Dégremont, Soletchnik and Boudry (2010) reported that triploidy did not confer any survival advantage in juvenile Pacific oyster under laboratory conditions, whereas Boudry, Dégremont and Haffray (2008) found higher survival rates in triploids (than diploids) following deployment in the field. It should be noted that for both studies, the triploids were obtained by crossing selected diploid females with unselected tetraploid males.

Hand, Nell and Maguire (1998) reported variable survival in triploid Sydney rock oysters (*Saccostrea glomerata*) produced by chemical induction compared to diploids at multiple farm sites. They suggested that this variability between ploidy was possibly the result of different environmental conditions of temperature and food availability between sites.

TABLE 2

Summary of studies and associated triploid induction methods comparing performances of both Pacific (*Crassostrea gigas*) and American cupped (*Crassostrea virginica*) triploid oysters (larvae, spat, adults) with their diploid counterparts. Performances include triploid success rates, survival and/or disease resistance compared with diploids, and growth (generally live weight) compared with diploids

Study	Method of triploid induction	Species	Life stage	Triploid success (%)	Survival rate/Disease resistance (vs 2n)	Growth rate (vs 2n)
Stanley, Allen & Hidu, 1981	MI induction (CB)	*C. virginica*	Larvae	59	Higher than 2n	N/A
Gérard *et al*., 1994	MI induction (6-DMAP)	*C. gigas*	Larvae	85	64% to D-larvae. Of those 19% to competency	Slower than 2n
	MII induction (CB)	*C. gigas*	Larvae	95	36% to D-larvae. Of those 11% to competency	Same rate as 2n
Gérard *et al*., 1999	MI induction (CB)	*C. gigas*	Larvae	33.5 ± 14	N/A	Not clear in larvae; Higher in spat
	MI induction (6-DMAP)	*C. gigas*	Larvae	18.3 ± 17	Lower D-yields than 2n (25% vs 51%)	Not clear; Higher in spat
Desrosiers *et al*., 1993	MII induction (6-DMAP)	*C. gigas*	Larvae	90	Lower than 2n	Equivalent to 2n

TABLE 2 (CONTINUED)

Study	Method of triploid induction	Species	Life stage	Triploid success (%)	Survival rate/Disease resistance (vs 2n)	Growth rate (vs 2n)
Gérard *et al*., 1999	MII induction (CB)	*C. gigas*	Larvae	84.3 ± 14.1	N/A	Not clear
	MII induction (6-DMAP)	*C. gigas*	Larvae	84.5 ± 13.8	Lower D-yields (34% vs 51%)	Not clear
Guo, DeBrosse & Allen, 1996	Mated Rutgers	*C. gigas*	Larvae	100	Similar to 2n	Higher than 2n
Vignier, 2017 Vignier *et al*., 2024	MII induction (6-DMAP)	*C. gigas*	Larvae	>95	Lower D-yields (~50% of 2n)	Faster than 2n
Vignier *et al*., 2024	Mated	*C. gigas*	Larvae	100	Similar to 2n	Higher than 2n
Cheng *et al*., 2024	Mated	*C. gigas*	Larvae	100	D-yields equivalent; Lower survival than 2n	Equivalent to 2n
Stanley, Hidu & Allen, 1984	MI induction (CB)	*C. virginica*	Adult	61	N/A	Faster than 2n
	MII induction (CB)	*C. virginica*	Adult	72	N/A	Same rate as 2n
Allen & Downing, 1986	MII induction (CB)	*C. gigas*	Adult	96	Higher than 2n	Higher
Matthiessen & Davis, 1992	MII induction (CB)	*C. virginica*	Spat to adult	>99	Higher than 2n	Higher
Guo, DeBrosse & Allen, 1996	Mated Rutgers	*C. gigas*	Spat to adult	100	N/A	Higher
Goulletquer *et al*., 1996	MII induction (CB)	*C. gigas*	Adult	66	Lower than 2n	Higher
Wang *et al*., 2002	MII induction CB	*C. gigas*	Adult	82–94	N/A	Larger (+26%)
	Mated Rutgers			100		Larger (+14%)
Garnier-Géré *et al*., 2002	MII induction (6-DMAP)	*C. gigas*	Adult	92	Similar	Faster
Boudry *et al*., 2008	Mated	*C. gigas*	Adult	100	Higher survival	N/A
De Decker *et al*., 2011	MII induction CB	*C. gigas*	Adults	>95	Lower (Winter)	N/A
	Mated				Higher (Summer)	
Dégremont *et al*., 2010	Mated	*C. gigas*	Juveniles	100	Similar to higher	
Dégremont *et al*., 2012	Mated Rutgers	*C. virginica*	Adult	100	Equivalent to higher	Higher (+88%)
Dégremont *et al*., 2014	MII induction CB	*C. gigas*	Spat	N/A	Similar resistance & survival	N/A
	Mated			100		
Pernet *et al*., 2012	Mated	*C. gigas*	Adult	N/A	Higher	N/A
Azéma *et al*., 2016	MII induction CB	*C. gigas*	Spat	N/A	Lower resistance	N/A
Callam *et al*., 2016	Mated Rutgers	*C. virginica*	Adult	100	3n = Increased resistance to Dermo	Higher
Ibarra *et al*., 2017	Mated	*C. gigas*	Adult	100	Equivalent	Higher (+18-70%)
Guévélou *et al*., 2019	Mated Rutgers	*C. virginica*	Juveniles	>98	Equivalent	N/A
Houssin *et al*., 2019	Mated	*C. gigas*	Juveniles-Adult		Lower	N/A
Wadsworth *et al*., 2019	Mated Rutgers	*C. virginica*	Adult	N/A	Lower	Higher
Matt *et al*., 2020	Mated Rutgers	*C. virginica*	Spat	>90	Equivalent to lower	Higher
Li & Li, 2022	Mated Rutgers	*C. gigas*	Adult	100	Lower (heat stress)	N/A
George *et al*., 2023	MI induction (T °C)	*C. gigas*	Adult	N/A	Lower (multi-stress)	N/A
					64% vs 85% in 2n	
Cheng *et al*., 2024	Mated	*C. gigas*	Adult	100	Higher	Higher (+25%)
Vignier *et al*., 2024	MII 6DMAP	*C. gigas*	Adult	>95	Equivalent to 2n	Higher (+20%)
	Mated Rutgers			100	Equivalent to 2n	Higher (+40%)

3n = triploid; **2n** = diploid; **4n** = tetraploid; **CB** = Cytochalasin B; **6-DMAP** = 6-Dimethylaminopurine; **MI** = Meiosis I; **MII** = Meiosis II; **N/A** = not available.

4.2.3 Disease-related mortality

Disease-related mortality differences between diploids and triploids vary. Hand, Nell and Maguire (1998) found that triploid Sydney rock oysters performed better against the parasite *Bonamia roughleyi*, the cause of winter mortality. In contrast an earlier similar study on winter mortality found no difference in mortality rates of Sydney rock oysters between ploidy levels (Nell *et al*., 1994).

The literature is also not conclusive regarding the comparative survivorship of triploid and diploid American cupped oysters (*C. virginica*) in the presence of *Perkinsus marinus* (or "Dermo" Perkinsosis) and/or *Haplosporidium nelsoni* (or MSX). Matthiessen and Davis (1992) found that chemically induced triploids had higher survival than diploids across three sites despite greater MSX infections. Meyers *et al.* (1991) exposed oysters to the Dermo causing parasite and did not find a difference in survival between diploids and triploids despite 100 percent infection rate. Callam, Allen and Frank-Lawale (2016) found, however, that triploid oysters had increased resistance to the parasite. Dégremont *et al.* (2012) found no difference in Dermo infection intensity between American cupped oysters with differing ploidy status although triploid oysters experienced 34 percent less mortality than diploids in the York River, Virginia (United States of America) where both Dermo and MSX are endemic.

For Pacific oysters, survival from the *Ostreid herpesvirus-1* (OsHV-1) was improved in young triploids during the summer and fall, but not during spring (Pernet *et al.*, 2012). Dégremont *et al.* (2014) examined the effects of ploidy on Pacific oyster spat mortality induced by a field-challenge with OsHV-1. Their results showed that virus-associated mortalities were similar between diploids and triploids (62 and 59 percent, respectively), with no apparent differences in triploid progenies derived from chemical induction (CB during meiosis II) or from mating of diploids with tetraploids.

Opportunistic bacterial pathogens frequently affect the oyster aquaculture industry and high summer temperatures usually exacerbate their virulence leading to summer mortality events (Destoumieux-Garzon *et al.*, 2020). As a result of their reduced reproductive potential, it has been hypothesized that triploids may be able to better tolerate pathogenic bacteria such as *Vibrio*. De Decker *et al.* (2011) conducted a repeated experimental infection of diploid and triploid Pacific oysters with *Vibrio*, at different times during the normal diploid reproductive period. They found that mortality rates were equivalent between diploids and triploids; however, triploids showed lower mortality during the reproductive summer season and higher mortality during the winter, highlighting the importance of sexual maturation in *Vibrio* infection processes. In contrast, Azema *et al.* (2016) demonstrated that triploid Pacific oyster spat produced by chemical induction using CB were significantly more susceptible to experimental infections with the pathogenic *Vibrio aestuarianus* than their diploid full siblings.

Current knowledge of the ability of triploids to tolerate diseases remains fragmented with no clear triploid advantage yet established. The influence of sterility associated with triploidy, and the additive and non-additive genetic variance of these traits on disease tolerance need to be further studied, notably to determine whether increased energy reserves (glycogen) may enable triploid oysters to better cope with, and/or tolerate infectious pathogens.

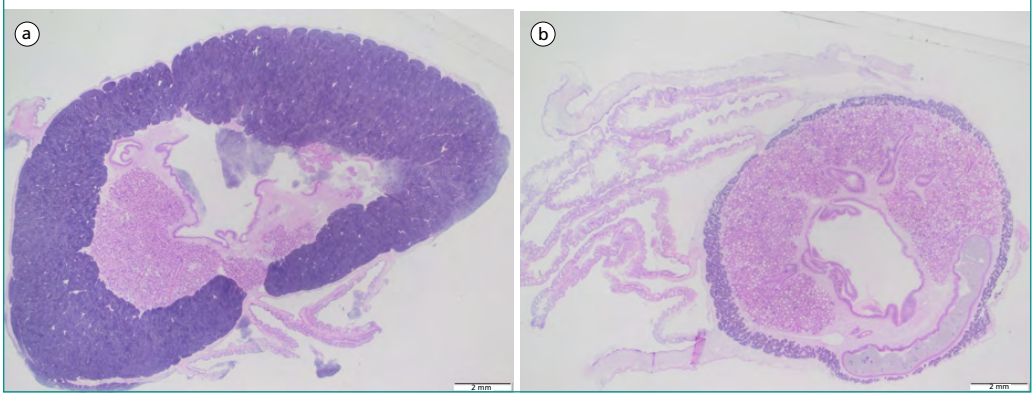
5. Challenges in triploid production

5.1 REPRODUCTIVE POTENTIAL

The reduced ability of triploid oysters to undergo gametogenesis has been well documented. Gametogenesis is not, however, fully inhibited (Figure 15) and there are some indications that environmental conditions such as elevated water temperatures or phytoplankton availability may allow gonad maturation of triploid oysters (Ibarra *et al.*, 2017; Normand, Pennec and Boudry, 2008). In addition, assuming that reproductive potential is a heritable trait, there have been growing concerns about the selection of triploid females with high fecundity to produce tetraploid stocks. The use of tetraploids (i.e. produced from a few fertile triploid females), in addition to limiting genetic diversity, may present a risk of producing subsequent triploids that show comparable or even higher fecundity than their triploid grandmothers; this may lead to the possibility of introgression of triploid gametes into the natural oyster population. Estimating heritability of the reproductive potential of triploid oysters and the associated viability of their gametes seems critical.

FIGURE 15

Histological cut showing a (a) typical diploid oyster with gonadal area in blue (covering ~80% of total body area) and (b) a triploid oyster with reduced gonadal area (covering ~10% of total body area). These animals were sampled during the peak of the reproductive season



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A study by Guo and Allen (1994b) justified the concern for the potential introgression by triploids; these authors investigated the reproductive potential of triploid Pacific oysters obtained from induction by CB treatment during meiosis II. They found that 78 percent of the studied triploids produced gametes, and that female triploids yielded an average fecundity equivalent to 2 percent of female diploid fecundity (Table 3). Moreover, gametes from triploids were viable and functional, demonstrated during the fertilisation of triploid eggs with diploid sperm, resulting in a 32 percent D-larval yield. However, the final survival of spat from fertilised eggs was much lower than standard, estimated at 0.046 percent (Guo and Allen, 1994b).

Likewise, Gong *et al.* (2004) studied the reproductive potential of Pacific oyster triploids obtained by crossing diploid females with tetraploid males (Rutgers method) and found that gametes were present in 98 percent of all triploids sampled, while average fecundity per female reached 13.4 percent of that of diploids (Table 3). When triploid eggs were crossed with diploid sperm, relatively high fertilisation rates

TABLE 3
Summary of studies and associated triploid induction methods comparing reproductive potential and cytogenetic instability (or chromosome losses assessed in either tetraploid parents or triploid progeny) in both Pacific (*Crassostrea gigas*) and American cupped (*Crassostrea virginica*) triploid oysters with their diploid counterparts. Reproductive potential data include fertility (presence of gamete), fecundity, gonadal occupation

Study	Method of triploid induction	Species	Reproductive potential	Cytogenetic instability
Guo & Allen, 1994b	MII induction CB	*C. gigas*	78% have gametes Fecundity: 2% of 2n	N/A
Allen *et al*., 1996	Mated Rutgers	*C. virginica*	N/A	Present with mosaic 3n
Jouaux *et al*., 2010	Mated IFREMER	*C. gigas*	25% have gametes Fecundity: 3% of 3n females	N/A
Gong *et al*., 2004	Mated Rutgers	*C. gigas*	98% have gametes Fecundity: 13.4% of 2n	N/A
McCombie *et al*., 2005a	Rutgers 4n	*C. gigas*	N/A	High mosaic
Normand *et al*., 2009	MII CB Mated IFREMER	*C. gigas*	GO: 47% of 2n GO: 47% of 2n	N/A
Suquet *et al*., 2016	Mated IFREMER	*C. gigas*	42% have gametes Fecundity: 0.5–1.5% of 2n	N/A
Zhang *et al*., 2010	Mated Rutgers	*C. virginica*	N/A	High reversion (offspring)
Matt & Allen 2014	Rutgers 4n Mated Rutgers	*C. virginica*	N/A	High mosaicism in 4n Moderate in 3n progeny
Benabdelmouna & Ledu, 2015	IFREMER 4n Rutgers 4n	*C. gigas*	N/A	Moderate (25% reversion) High (45% reversion)
Peachey & Allen, 2016	Mated Rutgers	*C. virginica*	Fecundity: 1.2% of 2n	N/A
de Sousa *et al*., 2016	Mated Rutgers	*C. virginica*	N/A	High in 3n progeny
Matt & Allen, 2021	Mated Rutgers	*C. virginica*	19% have gametes	N/A
Cheng *et al*., 2024	Mated	*C. gigas*	Fecundity: 23–41% of 2n	Low (>96% 3n)
Vignier *et al*., 2024	MII 6DMAP Mated Rutgers	*C. gigas*	GO: 26% of 2n GO: 26.5% of 2n	N/A

3n = triploid; **2n** = diploid; **4n** = tetraploid; **CB** = Cytochalasin B; **6-DMAP** = 6-Dimethylaminopurine; **MII** = Meiosis II; **GO** = Gonadal Occupation; **N/A** = not available.

(85 percent), and D-larval yields (21 percent) were obtained. In this case, long-term survival rates from fertilised eggs to 2-month-old spat were higher than those obtained by Guo and Allen (1994b), reaching 0.314 percent (Gong *et al*., 2004).

These results concur with more recent observations by Jouaux *et al*. (2010) on triploid oysters obtained by crossing diploid females with tetraploid males (direct IFREMER method); these authors report that 25 percent of all triploids sampled presented mature gametes, and 3 percent of the females were highly fecund (Table 3). Jouaux *et al*. (2010) subsequently proposed to categorize triploid animals displaying mature gametes as α-triploids, and animals with locked gametogenesis and immature gametes as β-triploids. Normand *et al*. (2009) compared the reproductive potential of triploid progenies produced from chemical induction and mated tetraploid x diploid (IFREMER method) with diploid relatives. Gonadal occupation was two times higher in diploids than in the two triploid groups, but equivalent (11.8 percent) in chemically induced triploid females and mated triploid females. Suquet *et al*. (2016) further quantified the reproductive potential of mated triploid and found that 42 percent of triploid Pacific oysters produced from diploid × tetraploid crosses (IFREMER method) produced gametes, with triploid sperm production of 1.5 percent compared to wild diploids, and average triploid female fecundity equivalent to 0.5 percent of wild

diploid (Table 3). The authors estimated the reproductive potential of triploid Pacific oysters (triploid × triploid crosses) at 0.06 percent of wild diploids.

In conclusion, the reproductive effort of triploid females shows some variability with contrasted relative fecundity (13 percent for Rutgers method *vs* 0.5 percent for IFREMER method *vs* 3 percent for chemically induced meiosis II triploids) which appears to be related to the method of production of triploids (Gong *et al.*, 2004; Suquet *et al.*, 2016; Vignier *et al.*, 2024).

5.2 CYTOGENETIC INSTABILITY

One disadvantage of polyploidy manipulation is the incidence of chromosome instability reported for both tetraploids and their triploid offsprings (Allen *et al.*, 1996; de Sousa *et al.*, 2016; Matt and Allen, 2014; Zhang *et al.*, 2010) (Table 3). Indeed, tetraploids were shown to undergo cytogenetic mechanisms of reversion (heteroploid mosaics) resulting in the loss of entire sets of chromosomes (McCombie *et al.*, 2005a; Matt and Allen, 2014) and aneuploidy (Zhang *et al.*, 2010). The use of a heteroploid mosaic parent led to high levels of aneuploids in the resulting offspring of *C. virginica* (Matt and Allen, 2014; de Sousa *et al.*, 2016) (Table 3). In contrast, Benabdelmouna and Ledu (2015) reported lower occurrence of aneuploid or reversion cases in tetraploid Pacific oysters produced using the direct IFREMER method (25 percent *vs* 45 percent with the Rutgers method) (Table 3). This inability of tetraploids to maintain genomic stability was shown to increase with age and/or in stressful environmental conditions.

5.3 IMPLICATIONS FOR BREEDING

Tetraploids have normal gonad development and fertility, comparable to diploids (Guo, DeBrosse and Allen, 1996); therefore, tetraploid founding populations can be crossed with each other to produce more tetraploids. It is important to ensure that tetraploid founder's and their pedigree information is curated and managed appropriately to avoid accidental crossing of related individuals (leading to inbreeding) and to ensure that the genetic diversity of any established tetraploid programme is adequately maintained.

Because tetraploids are mostly obtained from a few fertile triploid females, in addition to low efficiency of the induction treatment which results in a small population of tetraploids to be perpetuated, the genetic diversity of tetraploids is generally limited. One method, developed by McCombie *et al.* (2005b) and referred as the complementary method, allows lines of tetraploids to be maintained by fertilizing diploid eggs with tetraploid sperm and then suppresses the release of PB2 to generate tetraploid larvae. This approach can help to diversify and broaden the genetic background of tetraploid populations since 50 percent of the genetic material in the tetraploids produced by this approach comes from the diploid females. It is also possible to obtain high heterozygosity and maintain high genetic diversity of tetraploid populations through sub-hybridization between different species and generations of tetraploids (allotetraploids) (Yue *et al.*, 2024; Zhang, Qin *et al.*, 2022).

As a result, mated triploid production has a key drawback over artificially induced triploids that needs to be taken into consideration when integrating triploid production into a selective breeding programme. It can be a lengthy process to incorporate genetic gain into a tetraploid breeding programme (minimum 4 years). Even using the direct tetraploid induction method developed by IFREMER, it still requires that tetraploids are produced, on-grown and then maintained for use in mated tetraploid production. Using the indirect Rutgers method requires an intermediate generation of triploids to be produced and for desired crosses to produce sufficient gametes for them to be used in tetraploid production. Depending on the outcomes, a dual breeding programme (diploid and tetraploid) will be required. In contrast, the direct triploid induction method enables genetic gains to be incorporated faster, directly from selected parents

in a diploid breeding programme into commercial triploid production (within 2 years).

The genetic contribution to a triploid offspring may also be a critical consideration. Chemically induced triploids have one set of chromosomes from their father and two from their mother whereas mated triploids have two sets of chromosomes from the tetraploid father and one from the diploid mother. In both situations, the choice of parental stock and the differing contributions of the two parents may influence performance. These unequal parental contributions can have implications particularly when a small effective population size is used for spawning, which can increase the risk of inbreeding (Miller *et al*., 2016).

To date, there has been very little research looking at genetic selection of tetraploid oysters to produce mated triploids and it is a complex subject requiring additional effort.

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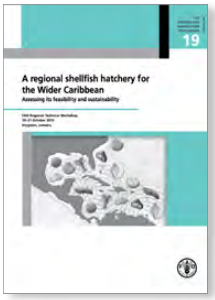

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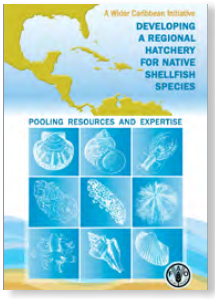

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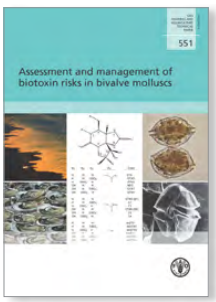

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Pacific oyster farming – A practical manual

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Glossary

Allele	One of two or more versions of a genetic sequence at a particular region (or locus) on a chromosome. An individual inherits two alleles for each gene, one from each parent.
Aneuploids	Organisms or cells having abnormal numbers of chromosomes, caused by chaotic segregation of chromatids. Aneuploids are not viable.
Diploid	Organisms that have two sets of chromosomes in each cell.
D-larva	Early larval stage of bivalves, swimming shelled larva with a characteristic "D" shape, also known as straight hinge veliger larva.
Embryo	Early stage of development resulting from an egg fertilised with a spermatozoon in bivalves, prior to larval stage.
Event	In flow cytometry, an event is defined as a single particle or cell detected by the instrument.
Fecundity	The ability of an organism to produce an abundance of offspring.
Gamete	A mature reproductive cell which is capable of fusing with a cell of similar origin but of opposite sex to form a zygote from which a new organism can develop. Gametes normally have a haploid chromosome content. In animals, a gamete is a sperm or egg; in plants, it is pollen, spermatic nucleus, or ovum.
Gametogenesis	Biological process by which female and male germ cells mature to form eggs/oocytes and sperm.
Genotype	The genetic make-up of an organism at the locus (or loci) that produces a specific phenotype. The organism is either homozygous or heterozygous at each locus; the sum of the total genetic information in an individual or the genetic constitution of an individual with respect to genetic loci under consideration.
Heritability	The degree to which a given trait is controlled by inheritance, as opposed to being controlled by non-genetic factors.
Heterozygosity	A general indicator of genetic diversity. Heterozygous refers to having different alleles for a particular trait.
Flow cytometer	A technology that simultaneously measures and then analyses multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light.
Larva	In bivalves, life stage starting from embryos to settlement.

Metaphase	Second stage of cell division by mitosis and meiosis where condensed chromosomes align on the metaphase plate in the middle of the cell, facilitated by spindle fibres. In meiosis, metaphase occurs twice. Metaphase I encompass the alignment of paired chromosomes along the centre, ensuring that two complete copies of chromosomes are present in the resulting two daughter cells. In Metaphase II, the two daughter cells have their meiotic spindles start to draw chromosomes to the metaphase plate again, before division takes place without any DNA replication.
Meiosis I	Initial phase of meiosis where homologous chromosomes separate, resulting in two haploid cells with duplicated chromosomes. This is a reductional division.
Meiosis II	Second phase of meiosis where sister chromatids segregate, resulting in four cells, each with a single copy of each chromosome in the set. This is an equational division.
Mosaics	Cells having lost entire sets of chromosomes to become heteroploidy mosaics through a process called reversion. Mosaic organisms comprise a mixture of both "diploid" and "triploid" cells.
Oocyte	An immature ovum or an egg that is not fertilised.
Ploidy analysis	It involves staining the nuclei of cells with a dye (DAPI usually) that binds to the DNA. Measurements of DNA contents in samples is then achieved using a flow-cytometer.
Polar body	Small cellular byproduct of the meiotic division of an egg/oocyte. They are extruded during meiosis I (polar body 1 or PB1) and meiosis II (polar body 2 or PB2) and contain either a subset of bivalent chromosomes (PB1) or a haploid set of chromatids (PB2).
Prophase	First stage of cell division by mitosis and meiosis where chromosomes condense. In meiosis, prophase occurs twice resulting in prophase I and prophase II. Prophase I is the most complex phase in all of meiosis because homologous chromosomes must pair and exchange genetic information (genetic recombination from chromosomal cross-over). Prophase II is very similar to mitotic prophase.
Settlement	Physiological process in which bivalve larvae change their planktonic behaviour to seek for a suitable substrate for attachment.
Spat	Newly settled bivalve larvae also called seed or post-larvae in bivalves.
Stripping	Sacrificial method to collect gametes consisting of an incision of the gonad.
Tetraploid	Organisms that have four sets of chromosomes in each cell.
Triploid	Organisms that have three sets of chromosomes in each cell.
Triploid rate	The percentage of a given life stage (e.g. D-larvae, spat), classified as triploid (as opposed to aneuploid, diploid or tetraploid).

Appendices

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Appendix 1
Procedure for conditioning broodstock

Most bivalves undergo an annual reproductive cycle that involves a period of gametogenesis, followed by a single or several spawning events, which in turn is followed by a period of gonad recovery (Utting and Millican, 1997). Pacific oysters are a temperate climate species and typically spawn naturally during the summer months (Pouvreau *et al*., 2006). To ensure that the direct triploid induction method yields the best triploid spat output, it is recommended to use mature adults (or broodstock) in prime reproductive condition.

For most bivalve species, microalgal food supply and seawater temperature are the most important factors determining the timing and extent of gametogenesis. By controlling temperature and food supply, hatcheries can control gametogenesis and synchronise the reproductive development of broodstock: this process is referred to as "broodstock conditioning". Conditioning broodstock is essential in the provision of improved quality and quantity of gametes for out-of-season large-scale production of larvae. Broodstock conditioning procedures have been well described for the Pacific oyster (e.g. Helm, Bourne and Lovatelli, 2004).

Briefly, *C. gigas* broodstock are brought into the hatchery during the winter months when they are rich in glycogen – an energy reserve essential for early gametogenesis. They are held in seawater at a temperature that is near the lower end of their tolerance range (8–10 °C) and fed a daily ration of 1–3 percent algae per oyster (dry weight) until 8–10 weeks prior to spawning (Figure A1.1). Algal feed generally consists of a mixture of multiple hatchery-grown microalgal species and naturally occurring microalgal assemblages grown in earthen ponds. This cold conditioning will typically last between 4 and 6 weeks but could be as long as several months if oysters are brought into the hatchery during the previous winter. The initial condition of oysters is very important, and glycogen reserves can be increased by extending the cold conditioning period. Insulated containers such as coolers are generally used to provide a temperature-stable environment during cold-conditioning.

Oysters are then warmed up at a rate of 0.5–2 °C/day to reach 21–22 °C and maintained at this temperature for 8–10 weeks on a daily ration of 5–8 percent algae per oyster (dry weight). The length of this second conditioning phase may be reduced during natural spawning season, when collected broodstock are at a more advanced gametogenic stage. Once ripe and ready to spawn, oysters are at risk of being triggered by small disturbances (e.g. small temperature fluctuations, changes in algal feed, movement, and desiccation) and should not be touched. Oysters can remain in spawning condition for several weeks but should be used as soon as possible to reduce the risk of spontaneous natural spawning before induction can take place. To maximize triploid success and larval output, broodstock should be supplied with a diverse conditioning diet. For example, broodstock should be fed a mixture of naturally occurring microalgal species previously grown in earth ponds (Figure A1.2), rather than monospecific cultures of hatchery-grown microalgal food (Vignier and Adams, unpublished data). Nonetheless, if pond algal production is low during winter months, hatchery-grown monospecific microalgal cultures and/or commercial feeds are added as a supplement.

FIGURE A1.1
Flow-through 100-L tank used for broodstock conditioning
Between 30 and 50 oyster specimens are maintained in the system and fed a mixture of hatchery-grown monospecific microalgae and naturally occurring microalgal assemblages



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FIGURE A1.2
Example of closed system earth ponds (500 m^3 capacity), bloomed with fertilisers are used to grow naturally occurring microalgal assemblages as food for oyster spat and broodstock



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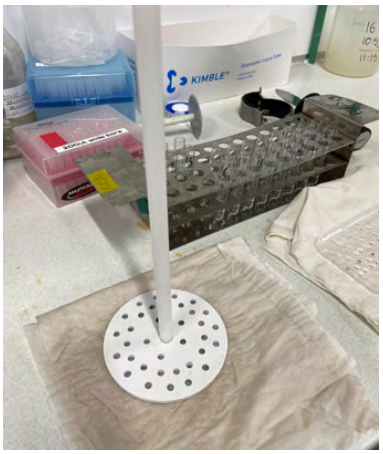
Appendix 2
Procedure for counting eggs/larvae

1. EQUIPMENT/REAGENTS

- Egg or D-larvae sample
- Compound or inverted microscope with appropriate magnification (e.g. 100× to 200×)
- Pipettors (1 000 µl; 200 µl; 20 µl) and tips
- Seawater or FSW (filtered, UV treated and EDTA-treated)
- Kimble® borosilicate glass test tubes (6 ml)
- Lugol's Iodine (5%)
- Counting chamber (Sedgewick rafter) or similar
- Plunger (Figure A2.1)
- 24 wells Tissue Culture Plates
- Sterile glass beaker

FIGURE A2.1
Plunger



2. METHOD

- Prepare a counting chamber/slide (or a well plate lid) and label accordingly.
- Prepare two Kimble® tubes per count and label adequately (Figure A2.2a).
  - Add 900 µL of FSW into the first tube
  - Add 400 µL of FSW into the second tube

- After stripping and washing on 55 µm screen, suspend eggs in a beaker (usually 900 ml of seawater depending on the number of eggs collected).
- Mix gently the egg solution using a plunger (Figure A2.2b).
- Collect a 100 µl subsample and add it to the first tube (containing 900 µl FSW).
- Change pipette tip.

- Mix the first tube while taking a 100 µl subsample and add it to the second tube (containing 400 µl FSW).
- Change pipette tip.
- Mix the second tube and take three subsamples of 20 µl from the second tube and place gently on a counting slide/well plate lid (Figure A2.2c).
- Count the eggs in each droplet under magnification (Figure A2.2d).
- If counting D-larvae, add a drop of diluted Lugol's iodine to fix the larvae prior counting (Figure A2.2e).
- Determine average number of eggs in three drops.
- To calculate the total number of eggs, calculate the number of eggs per ml and multiply this by the dilution factor and volume of FSW containing the eggs in the beaker. For example:
  - Average of 46 eggs per drop from 900 ml initial volume would be:
    46 × 50 × 50 × 900 = 103.5 million eggs total number.

    Note: if you have low numbers of eggs, you can skip a dilution step/the second tube (sample 20 µl directly from the first tube) – you will then only multiply by 50 (e.g. 46 × 50 × 900).

FIGURE A2.2
Gamete/D-larvae counting workflow



a) Prepare several tubes for serial dilution of gametes/larvae (2 to 3 tubes can be used based on density of sample);
b) Homogenise egg/larvae solution using a plunger and collect a subsample;
c) After dilution, collect several diluted subsamples (from tube 3) and place on plate;
d) Proceed to counting under magnification;
e) Example of D-larvae under magnification (x10).

Appendix 3
Procedure for preparing 6-DMAP solutions

1. EQUIPMENT/REAGENTS

- 6-dimethylaminopurine (6-DMAP) salt (CAS No. 938-55-6)
- Deionised water (Milli-Q 18 MΩ/cm)
- Sterile Schott type glass bottle (1 to 5 L)
- Analytical balance
- Hotplate magnetic stirrer and magnetic rod
- Filtered seawater (or FSW)
- Plastic container (e.g. 8 L)
- 15 µm screen (e.g. 250 mm Ø)
- Timer
- Gloves, eye protection (as per MSDS recommendation)

2. MAKING THE STOCK SOLUTION

- Use sterile glassware (e.g. 1 L Schott type glass bottle).
- Make a **0.05 M/L stock solution** by dissolving 6-DMAP salt in Milli-Q/deionised water
- Once 6-DMAP is weighed, add a magnetic stirrer into the glass bottle and add MilliQ water.
- Place glass bottle on a heated stir-plate and leave until fully dissolved (usually min 1–2 hours). Therefore, **prepare ahead of first fertilisation (at least 1 hour before)**.

3. MAKING THE WORKING/TREATMENT SOLUTION

- Use an 8 L clean container (Figure 8).
- Add 4 L of FSW at 25 °C. Generally, up to 50 million eggs can be induced effectively in 4 L of working solution.
  Note: for larger number of eggs to be treated, increase the volume of working solution.
- Add 9 mL of pre-made solution of 6-DMAP per litre of seawater (i.e. **36 ml/4 L**) to reach a final concentration of **450 µM/L** and mix.
- Set timer for 10 minutes.

Appendix 4
DAPI staining solution for ploidy analysis

1. REAGENTS

- TRIS hydrochloride, pH 7.5
- Magnesium chloride ($MgCl_2$)
- Triton X-100
- DAPI stock staining solution (1 mg/ml)
- Deionised water (Milli-Q 18 MΩ/cm)

2. MAKING THE STOCK BUFFER

0.2 M TRIS hydrochloride, 4 mM $MgCl_2$, 0.5% (v/v) Triton X-100

- Buffer should be prepared fresh.
- Prepare a 0.8 M TRIS hydrochloride solution by dissolving TRIS hydrochloride in deionised water and adjusting pH to 7.5.
- Separately prepare a 40 mM solution of $MgCl_2$ in deionised water.
- Combine 125 ml of 0.8 M TRIS hydrochloride, 50 ml of 40 mM $MgCl_2$, 2.5 ml of Triton X-100 and 322.5 ml of deionised water.

3. PREPARING THE WORKING STAIN SOLUTION

4 µg/mL DAPI solution

- Dilute DAPI stock staining solution 250 fold with stock buffer to obtain a working solution (e.g. add 400 µl of DAPI stock staining solution to 100 ml of stock buffer).

Adapted from:

de Laat, A.M.M., Göhde, W. & Vogelzang, J.D.C. 1987. Determination of ploidy of single plants and plant populations by flow cytometry. *Plant Breed.*, 99: 303–307.

Galbraith, D.W., Lambert, G.M., Macas, J. & Dolezel, J. 2001. Analysis of nuclear DNA content and ploidy in higher plants. *Curr. Protoc. Cytom.*, 7.6.1–7.6.22. Wiley Online Library. Doi:10.1002/0471142956.cy0706s02.

Appendix 5
List of chemicals

Please note that Material Safety Data Sheet (MSDS) should be consulted before use of each chemical agent. Appropriate personal protective equipment (PPE) should be worn.

6-DMAP	6-Dimethylaminopurine (CAS No. 938-55-6). A protein kinase inhibitor that interferes with the microtubule during cell division, thus inducing triploidy in oysters. Its low toxicity means it is used as an alternative to Cytochalasin B. Usage requires supervision by a veterinary and/or strict traceability as veterinary compounds.
CB	Cytochalasin B (CAS No. 14930-96-2). It is a cell-permeable mycotoxin that inhibits cytoplasmic division by blocking the formation of actin polymerization and interaction of actin filaments. It is very toxic and should be utilised with appropriate PPE, including a mask.
CyStain®	DAPI based UV ploidy solution. A proprietary reagent used to stain DNA in cells for ploidy level determination.
DAPI	4',6-diamidino-2-phenylindole (CAS No. 28718-90-3) is a fluorescent stain that binds strongly to DNA. As DAPI can pass through an intact cell membrane, it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells and therefore provides a marker for membrane viability. DAPI can also be used for fixed cell staining. Despite being labelled non-toxic in its MSDS, it is likely to have mutagenic and carcinogenic effects, so care should be taken in its handling and disposal.
DMSO	Dimethyl sulfoxide (CAS No. 67-68-5). An organic solvent used to dissolve Cytochalasin B. Toxic chemical.
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate (CAS No. 6381-92-6). A chelating agent generally used to bind to metal ions and remove heavy metals from seawater, hence reducing toxicity to the sensitive early life stages of bivalve.
Epinephrine	Epinephrine hydrochloride (CAS No. 329-63-5). Also known as Adrenaline is a neurotransmitter commonly used in shellfish hatcheries to induce the metamorphosis of bivalve, without the need for a substrate. This results in cultchless or single seed spat. Usage requires supervision by a veterinary and/or strict traceability as veterinary compounds.
Ethanol	Ethyl alcohol absolute (CH_3CH_2OH) (CAS No. 64-17-5). Used to disinfect surfaces, store tissue for later DNA extraction, or as a fixative for larvae.

Formalin	Formaldehyde is an organic compound (CAS No. 50-00-0) and a precursor to more complex compounds and materials. In its aqueous form, it is called Formalin, and is widely used to preserve or fix tissue or cells. In the hatchery, it is added to seawater at 1:10 to obtain buffered 10% formalin. Formalin is classified as a carcinogen and can cause respiratory and skin irritation upon exposure, so extreme care should be taken in its handling and disposal.
Lugol's iodine	Lugol's iodine fixative solution is made from Potassium Iodine (KI), Iodine (I2), and glacial acetic acid (CH_3COOH). A fixative commonly used to colour and fix bivalve larvae and aid for counting.
Magnesium chloride	Magnesium chloride is a salt with the formula $MgCl_2$ (CAS No. 7786-30-3). It has a wide array of practical uses (nutrition, medicine, cuisine, catalysis, wastewater treatment, etc).
Sodium carbonate	Na_2CO_3 or Soda ash (CAS No. 497-19-8). Used to buffer the pH of seawater and mitigate the detrimental effects of acidification on bivalve by improving shell calcification of the early embryo-development.
Triton X-100	Triton X-100 is a non-ionic surfactant (CAS No. 9002-93-1) commonly used as a detergent in laboratories. Due to its surfactant properties, it is used to lyse cells to extract protein or organelles, or to permeabilise membranes of living cells.
TRIS hydrochloride	TRIS is an organic compound commonly used as a component of buffer solution. The useful buffer range for TRIS (pH 7-9) coincides with the physiological pH typical of most living organisms. TRIS also inhibits enzyme activity via chelation of metal ions. It can also increase permeability of cell membranes.

Notes and observations

Naturally spawned oysters are typically diploid, each cell containing two sets of chromosomes. Diploid oysters exhibit seasonal variability in condition because they spawn during the summer months following a period of ripening as sea temperature increases. Often this coincides with the peak period for selling oysters in many countries. Triploid oysters contain three sets of chromosomes and because they are for the most part sterile, they can maintain condition throughout the year. As a result, triploids are "fatter" and preferred by consumers. Growth of triploids is generally much faster than diploids grown under the same conditions. For these reasons, many hatcheries around the world produce triploid oysters.

There are two main techniques to commercially produce triploid oysters in the hatchery. The first method, widely adopted around the world since the mid-1990s, makes use of another ploidy level, tetraploid (having four chromosome sets). Crossing male tetraploids with diploids females results in 100 percent triploid offspring. The second method, which is outlined in this technical manual, induces the triploid condition directly by treating fertilised diploid eggs with a chemical solution (6-DMAP). High triploid rates (>95% triploid) are typically obtained.

Following a brief overview of triploid oyster production around the world and the main benefits of growing triploids, this manual summarises the different induction methods available to hatchery operators to produce triploids and tetraploids. An extensively detailed methodology to produce large quantities of triploid Pacific oysters by direct chemical induction is provided. Overall methodologies on broodstock conditioning, larval rearing, feeding systems and mating tetraploid broodstock with diploid broodstock for all triploid spat production are also provided as a guide, but not the focus of this manual. Finally, a review of previous research comparing the physiological performance of triploids (produced by different induction methods) in relation to diploids is given to highlight the potential trade-offs of using either method for commercial production as well as to ensure the longevity of triploid use in oyster aquaculture.

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