PLOIDY MANIPULATION OF PENAEID SHRIMP TO CREATE REPRODUCTIVELY STERILE, FEMALE STOCKS FOR COMMERCIAL PRODUCTION

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SUMMARY

Domestication and selective breeding programs in a number of cultured penaeid shrimp species worldwide have substantially improved commercially desirable traits compared to wild stocks. Improvements in growth rates, feed conversion efficiency, disease resistance and survival have been achieved in the important aquaculture species including *Litopenaeus vannamei, Penaeus monodon, Fenneropenaeus chinensis* and *Marsupenaeus japonicus.* However, there are substantial economic investments associated with the development of genetically superior lines in breeding programs and the intellectual property associated with such lines requires protection in the absence of effective legal mechanisms. Various technical strategies have been developed to protect these genetic resources with limited success to date.

Currently, triploidy is the only method known to guarantee inhibition of reproduction in shrimp and thus confer genetic protection. In addition, triploidy can increase the proportion of females in some shrimp species which is commercially desirable, as females grow faster than males. Triploid *F. chinensis, M. japonicus* and *P. monodon* have been produced by inhibition of polar body I or II formation during meiosis. A number of treatment agents to induce triploidy in shrimp have been trialled, with greatest success achieved using chemical and thermal shocks. However, current triploid induction methods cannot produce triploids at high or absolute rates and are not practical for commercial adoption due to the challenges associated with treating entire spawnings and the high mortality associated with the necessary handling of fragile eggs and embryos.

Tetraploid induction has also been investigated, as mating of tetraploid and diploid broodstock could in theory produce all triploid progeny. Tetraploidy has been reported in *F. chinensis, M. japonicus, Fenneropenaeus indicus* and *L. vannamei* by preventing first cleavage with thermal shocks; however, no tetraploid embryos have been reared to postlarvae.

This study progressively trialled a range of novel techniques to improve triploid inductions in *M. japonicus* and *P. monodon* in an attempt to move this technology

towards commercialisation. This study also used alternative treatment agents in an attempt to produce viable tetraploids. Initially tetraploid inductions were attempted with cold temperature and hydrostatic pressure shocks that were applied to inhibit first mitosis. Tetraploid *P. monodon* were induced following cold shock treatments at temperatures between +1ºC and -2ºC; however, treatments were lethal to all embryos, both tetraploid and diploid. Tetraploid *M. japonicus* embryos were induced following hydrostatic pressure treatments between 24.1 MPa and 34.5 MPa. Despite investigating an extensive range of induction parameters, no tetraploid *M. japonicus* nauplii were produced, with all embryos, both diploid and tetraploid, dying following exposure to the pressure levels of 24.1-34.5 MPa, which were required to induce tetraploidy. Cytological analysis of pressure treated tetraploid embryos revealed that half of the cells from the 4-cell stage were anucleate, which was not resolved and thus development ceased.

Following the failure to produce viable tetraploids, this study then investigated the direct induction of triploidy through pressurisation in experimental sized chambers. Hydrostatic pressure successfully induced triploidy in *M. japonicus* and *P. monodon*, with triploid nauplii produced at high or absolute rates following treatment using optimal induction parameters. However, as handling of fragile eggs and embryos to perform experimental sized chamber inductions was required, significant reductions in hatching rate were observed as a result of physical stress. Finally, a large pressure chamber was engineered that would allow a shrimp to spawn inside the chamber enabling treatment of all the progeny with optimised pressure and eliminating the requirement to handle the fragile eggs and embryos. Triploid *M. japonicus* families were successfully induced at high or absolute rates following treatment at 17.2 MPa in the large chamber system.

This research supports the theory that tetraploidy may not be a viable option in penaeid shrimp for the purpose of breeding triploids for genetic protection. However, an improved method of direct triploid production on a large scale was developed. The production of triploid shrimp by using a large hydrostatic pressure chamber addresses some of the major constraints to commercial adoption of triploidy. Further refinement of this technique would be required to provide a robust and fail-safe method for commercial scale induction of triploidy, that might also increase the proportion of females in some species.

DECLARATION

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

Andrew Robert Foote

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CHAPTER 1

General Introduction

Global demand for seafood continues to grow at a rate exceeding population growth, despite production from traditional capture fisheries remaining relatively stable for the last decade (FAO 2010). Subsequently, aquaculture has become one of the fastest growing animal food-producing industries in the world to meet the demand for seafood, with aquaculture now accounting for almost half the world's supply (FAO 2010). Of the 126 million tonnes of seafood available for human consumption in 2009, two-thirds or 85.4 million tonnes was consumed in Asia, 42.8 million tonnes of which was consumed in China alone. In the next decade, total global production from both capture fisheries and aquaculture is expected to exceed that of beef, pork or poultry (FAO 2010).

Shrimp[1] are one of the most valuable and fastest growing cultured seafoods in the world, providing food, employment and a large export earner for many countries, particularly those with developing economies. Cultured shrimp production has almost tripled between 2001 and 2010, increasing from approximately 1.3 million tonnes to 3.8 million tonnes, with a value of US$16.7 billion in 2010. The most commonly farmed species is the Pacific White Shrimp, *Litopenaeus vannamei*[2], making up more than 70% or 2.7 million tonnes of the total shrimp production in 2010, with China the largest producer, farming 1.2 million tonnes of this species (Food and Agriculture Organisation of the United Nations, www.fao.org). The second most commonly farmed shrimp is the Black Tiger Shrimp, *Penaeus monodon*, with approximately 781,000 tonnes produced in 2010, Vietnam is the highest producer of this species, with 333,000 tonnes farmed in 2010 (Food and Agriculture Organisation of the United Nations, www.fao.org). In comparison, Australia's shrimp farming industry is small, with approximately 4,000 tonnes of shrimp produced in 2009 with a value of AU$70 million (Australian Prawn Farming Association, www.apfa.com.au). In Australia, production of *P. monodon*

[1] The term 'shrimp' rather than 'prawn' will be used as it the most accepted term used globally
[2] Species have been named following the *Integrated Taxonomic Information System* (www.itis.gov)

predominates, while the Banana Shrimp, *Fenneropenaeus merguiensis* and a small quantity of Kuruma Shrimp, *Marsupenaeus japonicus* are also farmed.

Many shrimp farms in Australia and around the world depend on the seasonal collection of wild broodstock to produce postlarvae that are then grown out to harvest age in ponds. Using wild shrimp stocks increases the level of risk for farmers due to the unreliable availability and performance of wild broodstock and their progeny, while also providing an avenue for disease introduction with the capability of causing mass mortality (Argue et al. 2002). Through domestication the shrimp industry can largely overcome the problems associated with wild sourced stocks (Hetzel et al. 2000; Coman et al. 2006). Furthermore, well managed domestication allows genetic selection for economically desirable traits to be implemented (Coman et al. 2006).

In Australia, significant advances have been made in selectively bred lines of *M. japonicus* and *P. monodon*. In *M. japonicus*, first generation selected stocks showed an increase in growth rate compared to wild stocks of approximately 10% by harvest age (Hetzel et al. 2000; Preston et al. 2004). There has also been considerable success in selective breeding of *P. monodon* in Australia, with significant increases in growth, survival, harvest yield and food conversion efficiency (Preston et al. 2009; Preston et al. 2010; Glencross et al. 2012).

The shrimp farming industry has identified genetic protection of improved lines as a research priority to protect the investment associated with breeding programs. Once the intellectual property of the improved lines is protected, the rest of the industry to may purchase the superior stock to grow-out and take advantage of genetic improvements. A second research priority is to increase the proportion of females stocked on farms. Female population are commercially desirable as penaeid shrimp are sexually dimorphic with the females growing significantly faster than males (Hansford and Hewitt 1994; Coman et al. 2004).

1.1. Genetic protection

While there are significant advantages in using domesticated broodstock that have been selectively bred as opposed to wild caught, there are substantial economic investments associated with developing superior lines in breeding programs. As a result, industry demands that economic investment be protected from unauthorised breeding before selling these improved lines to the rest of the industry, allowing controlled access to improved lines, allow supply and demand to be managed and to recover some of the investment made in genetic improvement programs. Therefore, research has investigated various strategies of protecting these genetic resources.

Currently the only option available for hatcheries to genetically protect shrimp has been a crude, indirect method, where hatcheries with improved stocks claim to sell only closely related broodstock, which if mated together may result in inbreeding depression. Inbreeding depression typically affects fitness-related traits such as survival, especially when cultured under sub-optimal conditions. In *L. vannamei*, the cumulative effects of inbreeding depression on hatch rates and survival in the hatchery meant that there was a considerable reduction in postlarval production (Moss et al. 2008). The problem with the inbreeding method is that inbred stocks are easily outbred by mating unrelated stocks from different hatchery companies or from wild stocks. In addition, the availability of a range of pedigree markers in several farmed species including *M. japonicus* (Moore et al. 1999), *P. monodon* (Jerry et al. 2006) and *P. vannamei* (Garcia and Alcivar-Warren 2007), allows genetic relatedness of individuals to be determined, allowing hatcheries to make informed mating decisions that facilitates outbreeding. Therefore, more reliable methods of guaranteeing genetic protection are needed with the primary focus on developing reproductively sterile shrimp.

Ionizing radiation (IR) has been used on a number of cultured animals to reduce or inhibit fecundity by damaging DNA (Coates et al. 2004), these include: Eastern Oysters, *Crassostrea virginica*; (Ardjosoediro et al. 2000; Ardjosoediro et al. 2001; Wagner et al. 2001), Rainbow Trout, *Oncorhynchus mykiss* (Konno and Tashiro 1982) and Atlantic Salmon, *Salmo salar* (Thorpe et al. 1987). IR has also been investigated as a means of reproductive sterilisation in the shrimp *M. japonicus*. However, the IR doses that did not result in shrimp death could not guarantee complete reproductive sterility in *M. japonoicus*, as some viable progeny were still produced (Sellars et al. 2005; Sellars and Preston 2005).

Triploid induction has been commonly used in several commercially aquacultured animals to reduce or inhibit reproductive development. In some cases triploidy conveys additional economically desirable traits such as faster growth, higher meat quality and sweeter taste (Allen and Downing 1991; Wang et al. 2002; Troup et al. 2005) in the case of oysters. Triploid production can be directly induced by preventing the first or second polar body from extruding during meiosis. A number of different treatment agents have been used to induce triploidy including chemical, heat, cold and hydrostatic pressure shocks in fish and shellfish.

The most successful treatment agent to induce triploidy varies between species, with differences observed even in closely related species: hydrostatic pressure has been more successful than heat shock at inducing triploidy at higher rates and survival in Coho Salmon, *Oncorhynchus kisutch* (Teskeredžić et al. 1993), in *O. mkyiss* triploid induction rates were similar but survival was higher in pressure induced triploids (Haffray et al. 2007) and in the case of Chinook Salmon, *Oncorhynchus tshawytscha* there was little difference in performance between heat and pressure induced triploids. It has been suggested that pressure and thermal shocks are more successful than chemical shocks in fish (Piferrer et al. 2009), while chemical shocks (cytochalasin B) are more successful in oysters (Nell 2002).

In penaeids, both chemical (cytochalasin B or 6-dimethylaminopurine) and thermal shocks (heat and cold) have yielded most success (reviewed by Sellars et al. 2010); however, pressure shock has not been trailed prior to this research project. Reproductive sterility of triploids has been reported though gonad analysis in the Chinese Shrimp, *Fenneropenaeus chinensis* (Li et al. 2003b), *M. japonicus* (Sellars et al. 2009) and *P. monodon* (Sellars et al. 2012a). Furthermore, both male and female *M. japonicus* (Sellars 2007) and *P. monodon* (Sellars et al. 2012a) triploids have been found to be incapable of reproduction, even when crossed with diploids. Triploid shrimp induction has been achieved by preventing polar body I and/or II, by using chemicals cytochalasin B in *F. chinensis* (Li et al. 2003c), 6 -dimethylaminopurine in *M. japonicus* (Norris et al. 2005; Sellars et al. 2006b) and *P. monodon* (Sellars et al. 2012b) and caffeine in *M. japonicus* and *P. monodon* (CSIRO unpublished). Heat shock has also been used in *M. japonicus* and *P. monodon* (CSIRO unpublished) and most recently cold shock induced triploidy in *P. monodon* (Wood et al. 2011). However, the problem with all direct methods of inducing triploidy in shrimp is an inability to reliably induce triploidy at high or absolute rates and additional problems arise when adapting small scale experimental methods to treat entire spawnings, which would be required for commercial adoption of triploidy.

One of the causes of low or variable triploid induction rates in shrimp has been attributed to difficulties in applying the required treatment at the precise stage of development to inhibit polar body I or II, due to asynchronous development of progeny (Li et al. 2003c; Norris et al. 2005). Penaeid shrimp egg activation occurs upon contact with seawater (Pongtippatee-Taweepreda et al. 2004; Rojas and Alfaro 2007), rather than following fertilisation and as eggs are released over a variable duration of up to several minutes, the progeny do not all develop at precisely the same time. It then follows that polar body formation will occur at slightly different times among the progeny and triploid induction treatments may only be successful for a proportion of the progeny.

One of the major issues with transferring current treatment agents and techniques to a larger scale is the requirement of handling progeny to apply and/or terminate the treatment of a whole spawning. Handling eggs and embryos early in development increases stress at the most fragile stages and consequently hatch rates of progeny that have been handled are inevitably lower than unhandled progeny. A recent attempt to treat entire spawnings of *P. monodon* eggs involved spawning the broodstock in mesh pens so they could be transferred in and out of a cold water bath for treatment (Wood et al. 2011). However, this method still resulted in handling stress and would increase complexity and labour inputs to the hatchery process. Alternative treatment methods/agents are therefore required to overcome each of the limitations outlined above before commercial adoption of triploidy can be considered.

An alternative method of producing triploids is to first produce tetraploid broodstock and then mate them with diploids to produce triploid offspring. This method of triploid production has been achieved in the Pacific Oyster, *Crassostrea gigas* (Guo et al. 1996; Wang et al. 2002) and Rainbow Trout, *Oncorhynchus mykiss* (Chourrout et al. 1986). Tetraploidy can be induced by preventing cytokinesis at the end of the first cell cycle, while allowing chromosome set doubling. Prevention of first cleavage has been achieved in a number fish and shellfish, typically using heat, cold and hydrostatic pressure to inhibit first cleavage.

Tetraploid penaeid shrimp have been induced using heat in *F. chinensis* (Li et al. 2003a) and *M. japonicus* (Sellars et al. 2006a) as confirmed by flow cytometry. Cytological analysis indicated that tetraploidy was also most likely induced in the Banana Shrimp, *Fenneropenaeus indicus* (Morelli and Aquacop 2003) and nuclear analysis also indicated that tetraploidy was most likely induced in *L. vannamei* (de Almeida Aloise et al. 2011). Tetraploid induction has been reported following cold shock in a single treatment in *M. japonicus* (Sellars et al. 2006a); however, the induction rate was low at 15%. Chemical (6-dimethylaminopurine) shock was also trialled without success in *M. japonicus* (Sellars et al. 2006a). However, in all cases no viable tetraploid postlarvae were produced and therefore the mating of tetraploid and diploid shrimp could not be achieved.

Cytological analysis of mitotic heat treated *F. indicus* (Morelli and Aquacop 2003) and *M. japonicus* (Foote et al. 2010) embryos revealed abnormalities when compared to normal development in control embryos. Instead of the usual bipolar mitotic spindle arrangement, polypolar spindle architecture was observed in treated embryos along with abnormal cell divisions and anucleate cells in some instances. In the case of *M. japonicus*, tetrapolar cell division from one to four cells coupled with an off-centre pronucleus resulted in anucleate cells in half the cells of each embryo from successful tetraploid inductions. As the anucleate trait was not resolved later in embryogenesis, development ceased (Foote et al. 2010). Similar abnormal polypolar spindle formations have also been observed in *O. mykiss* embryos following mitotic heat treatment; however, when the alternative treatment agent of hydrostatic pressure was used to inhibit mitosis, normal mitotic spindle formation was regenerated. Hydrostatic pressure induction is yet to be trialled in penaeid shrimp (or any other crustacean) to determine if the abnormalities mentioned above can be overcome and tetraploids produced.

1.2. Monosex populations

Monosex populations have been developed in the aquaculture industry to improve growth, survival, feed conversion ratio and production by taking advantage of sexual dimorphism, diversion of energy from gonad and courtship behaviour to growth, by reducing aggressive sexual/territorial behaviour and by avoiding undesirable impacts of sexual maturation on appearance and flesh quality (Trino et al. 1999; Beardmore et al. 2001; Dunham 2011). Monosex populations have been achieved in many commercially farmed animals such as the Giant Freshwater Shrimp, *Macrobrachium rosenbergii* (Karplus et al. 1992), mud crab *Sycylla* species (Trino et al. 1999), various tilapia (*Oreochromis*) species (Beardmore et al. 2001), Rainbow Trout, *Oncorhynchus mykiss* (Bye and Lincoln 1986; Arslan et al. 2010) and several salmonids (Donaldson and Hunter 1982; Baker et al. 1988; Fitzpatrick et al. 2005). Techniques used to create monosex stocks include sex-reversal, hybridisation, gynogenesis or androgenesis and polyploidy.

Penaeid shrimp are sexually dimorphic, with the females reported to grow at a significantly faster rate than males in several commercially farmed species including P. monodon, (Hansford and Hewitt 1994; Coman et al. 2005; Coman et al. 2010; Gopal et al. 2010) *M. japonicus* (Coman et al. 2004; Preston et al. 2004) and *L. vannamei* (Perez-Rostro et al. 1999; Pérez-Rostro and Ibarra 2003; Gitterle et al. 2005). In species such as *P. monodon* and *M. japonicus* this divergence occurs at a relatively early age and is significant by the time they attain harvest size , while in *L. vannamei* divergence in size may not be significant until after the age and size at which they are normally harvested (Perez-Rostro et al. 1999); all studies have indicated that the rearing environment, diet and/or husbandry techniques should also be considered in influencing growth rates. Therefore, the production of female stocks of shrimp is particularly desirable in *P. monodon* and *M. japonicus*, as stocking ponds with all females would increase both harvest yield and the size class.

1.2.1. Triploidy

Triploidy can increase the proportion of females in some farmed penaeids and it is the only known method to also confer reproductive sterility (Sellars et al. 2006b). Triploid *M. japonicus* induced through inhibition of polar body I (PBI) have been found to be mostly female, while100% female populations were achieved following inhibition of polar body II (PBII) (Sellars et al. 2009). Triploid *F. chinensis* induced by preventing both PB I and II at the same time have a 4:1sex ratio skewed to the female (Li et al. 2003b). In *P. monodon* one study claims a sex ratio of 2 females: 1 male in triploids (Pongtippatee et al. 2012); however, it is unclear if both PBI and PBII were inhibited and ploidy analysis did not meet the standards outlined by Sellars et al. (2010) (no internal control was included and insufficient cells were analysed). Another *P. monodon* triploid study (which did meet the required flow cytometry standards) found a sex ratio of triploids skewed slightly to the male; 1 female to 1.625 males when inhibiting PB II (Sellars et al. 2012b).

1.2.2. Sex-reversal

Sex-reversal has been achieved in over 50 species of fish from at least 15 families through exposure to steroid hormones (androgens for male production or estrogens for female production). Hormone's are typically administered via dietary supplementation; however, immersion or injection has also been used (Beardmore et al. 2001).

In penaeid shrimp, there are no reports of sex-reversal through exposure to steroid hormones. However, sex-reversal of *M. rosenbergii* has been achieved through the removal or implantation of the androgenic gland. Removal of the androgenic gland from immature male *M. rosenbergii* is reported to have resulted in sex-reversal to neofemales and similarly, implantation of the androgenic gland into immature female *M. rosenbergii* is reported to result in the development of a male reproductive system (Sagi and Aflalo 2005). Following crosses of neofemales with normal males, monosex populations of male *M. rosenbergii* could be produced (Rungsin et al. 2006). While a putative androgenic gland has also been localised in *M. japonicus* (Payen et al. 1982), *L. vannamei* (Campos-Ramos et al. 2006) and *F. chinensis* (Li et al. 2012). It has been postulated that a peptide hormone in the androgenic gland may control sexual differentiation; therefore, such peptides have been isolated and characterised in *P. monodon* (Mareddy et al. 2011) and *F. chinensis* (Li et al. 2012). However, in all penaeid shrimp that sex-reversal has been attempted, incomplete sex-reversal has resulted; with the apparent reversal of some sex-traits such as aspetasma developing in females (Mareddy Personal Communication). There is also a patent on using the androgenic sex hormone to influence the sex ratio in shrimp cultures and to set up monosex cultures (Staelens et al. 2009; Van Breusegem et al. 2009).

1.2.3. Hybridisation

In cultured animals, hybrids have been developed as a means of creating monosex populations among other desirable characteristics such as hybrid vigour for commercially available quantitative traits, sterility or to combine desirable characteristics from two species. One of the best examples of monosex hybrids has been reported in tilapias, where all male populations have been achieved, increasing the proportion of the faster growing sex (Pruginin et al. 1975).

In penaeid shrimp, hybrid crosses have been achieved between *P. monodon* and the Brown Tiger Shrimp, *Penaeus esculentus*, with hybrids displaying a skewed sex ratio towards males (0.86) (Benzie et al. 2001). As the absent or rare sex in hybrid progeny is likely to be the heterogametic sex (Haldane 1922); females are likely heterogametic in penaeids. Therefore, hybridisation may not be a suitable technique for producing all female populations of shrimp.

1.2.4. Gynogenesis/androgenesis

Artificial gynogenesis (all maternal inheritance) and androgenesis (all paternal inheritance), has been used to create monosex populations in a number of cultured fishes (Onozato 1984; Taniguchi et al. 1988; Hussain et al. 1993; Goudie et al. 1995; Johnstone and Stet 1995; Peruzzi and Chatain 2000), reviewed by Komen and Thorgaard (2007) . This is typically achieved by destroying/fragmenting the DNA content of either the sperm or egg through irradiation to ensure there is no paternal or maternal contribution and then fusing this treated gamete with an untreated egg or sperm to create a haploid embryo. Diploidisation can then be achieved in the same way that triploids and tetraploids are induced; by either inhibiting polar body extrusion (meiotic gynogens) or mitosis (mitotic gynogens).

In penaeids, the first steps towards gynogen production have been investigated in *F. chinensis* (Cai et al. 1995; Chen et al. 1997). Sperm were UV treated at different levels in an attempt to prevent paternal genetic contribution while stimulating development of the egg. However, there was no fertilisation and diploidy was not achieved, preventing successful development and hatching of embryos (Cai et al. 1995; Chen et al. 1997). Even if successful production of gynogens can be achieved in penaeid shrimp, it is unlikely that it could be used to produce all female populations due to the evidence that penaeid shrimp have a ZW sex determination system, with females as the heterogametic sex; several female-derived markers in *P. monodon* have been identified in complete linkage with sex (Staelens et al. 2008). Further indirect evidence supports the presence of a ZW system with hybrid shrimp sex ratios skewed to the male (Benzie et al. 2001), identification of female-derived markers appear to be only on the maternal genomic map in *M. japonicus* (Li et al.

2003d) and *M. japonicus* triploids are predominately or all female (Sellars et al. 2009).

1.2.5 Gene-regulation

The genetic basis for sex determination in penaeid shrimp is currently underway, with the aim of using gene regulation to control shrimp sex and fertility (Sellars and Preston 2008). Identifying genes responsible for controlling sex and fertility in shrimp is in its early research stages; with candidates under investigation that are typically based on genes found to have similar functions in *Drosophila*. The essential sex determination gene in *Drosophila*, *vasa* has been targeted as mutant female *Drosophila* lacking a functional *vasa* gene produces sterile embryos. In *M. japonicus* and *P. monodon vasa* orthologues, *Mj-vasa* and *Pm-vasa* respectively have been isolated; this gene may play a role in early germ cell specification providing a potential avenue for the production of sexually sterile *M. japonicus* and *P. monodon* (Callaghan 2011). Similarly, *vasa*-like genes have been isolated in other penaeid species, namely *L. vannamei*, (Aflalo et al. 2007); and *Sicyonia ingentis* (Hertzler et al. 2008).

Recently, a putative germ granule, termed the intracellular body (ICB) has been discovered in penaeid shrimps, firstly in *P. monodon* (Biffis et al. 2009) and then *M. japonicus* (Foote et al. 2010), *L. vannamei* and *Sicyonia ingentis* (Pawlak et al. 2010). It is hypothesised that the RNA rich ICB could play a role in germ line specification, as it is always observed in one of the two mesendoderm (ME) cells that eventually gives rise to the presumptive primordial germ cell (Pawlak et al. 2010). Further research is continuing to determine if the ICB segregates exclusively into the ME cell that gives rise to the presumptive primordial germ cell and determine the presence of sex-related genes mentioned above.

1.4. Aims of this research project

As significant advancements have been made in selectively breeding penaeid shrimp, genetic protection of these highly valuable lines is becoming increasingly urgent to allow the rest of the industry to take advantage of the improvements, while protecting the economic investment of companies which developed and produced the improved lines. Furthermore, as female penaeid shrimp grow faster than males, there would be significant commercial gains from stocking increased proportions of females. Currently, triploid induction provides the only short term avenue for guaranteeing genetic protection as well as producing all females in *M. japonicus*, potentially increasing the proportion of females in other farmed species. However, current methods of triploid production are not suitable for commercial adoption due to low or unreliable induction rates and difficulties treating entire families/spawnings without increasing mortality from handling stress.

Therefore, this project seeks to develop a method to produce triploid shrimp in a manner that addresses these major issues and attempts to progress triploid production closer towards a method that could be adopted by commercial hatcheries. The approach was to trial novel treatment agents and methods to induce triploidy; both indirectly though inhibition of first cleavage to induce tetraploidy, and also direct induction of triploidy though inhibition of PBII. The objectives of my research project were as follows:

1. Perform cold shock treatments to induce tetraploidy and determine if viable tetraploid postlarvae can be produced (Chapter 2).

2. Perform a range of hydrostatic pressure treatments to induce tetraploidy in *M. japonicus* and determine if the cytological abnormalities observed previously in heat treated embryos (Foote et al. 2010) are overcome with this alternative method (Chapter 3).

3. Perform a range of hydrostatic pressure treatments for direct induction of triploidy in *M. japonicus* Chapter 4).

4. To attempt direct induction of triploidy using hydrostatic pressure in *P. monodon* and to trial a large scale induction technique which could be adopted by commercial hatcheries (Chapter 5).

CHAPTER 2

Tetraploid inductions of *Penaeus monodon* using cold shock

A version[3] of this chapter has been published as:

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Abstract

Genetic protection of improved lines of Black Tiger Shrimp, *Penaeus monodon*, may be achieved through the reliable production of all triploid families, as triploid shrimp are reproductively sterile. The problem with current triploid induction methods is in producing 100% triploid progeny reliably and doing so on a commercial scale. All triploid offspring may be achievable by mating tetraploids with diploids, overcoming current limitations. However, tetraploidy in shrimp to date has been unsuccessful, with all tetraploids dying very early in development. As the induction method of heat was found to be lethal, this study investigated an alternative method using cold water to assess induction rates and lethality. Cold water treatments between -2°C and 14°C were applied at 18 min, 20 min and 22 min post-spawning detection for various durations with the aim of inhibiting first mitosis. Tetraploidy was induced in treatments at or less than 1°C; however, no nauplii hatched from these treatments. As no diploid or tetraploid nauplii hatched from any of the treatments below 3°C, it was determined that this cold water treatment method is not suitable for tetraploid production in *P. monodon*.

[3] Minor changes were made to the formatting, spelling (to 'Australian English') as well as standard common and genus names to allow consistency throughout this thesis

2.1. Introduction

The production of genetically improved lines of Black Tiger Shrimp, *Penaeus monodon*, has promoted research into methods of genetic protection, to prevent unlicensed breeding of improved stocks once they have been sold to other farms. The production of triploid *P. monodon* is one method currently under investigation. Triploid shrimp have been found to be reproductively sterile with greatly reduced gonad tissue, abnormal cellular organisation in the gonads and a lack of mature oocytes or spermatophores when compared to their diploid control siblings (Li et al. 2003b; Xiang et al. 2006). In reproductive performance trials, triploid *P. monodon* have also been shown to be incapable of producing viable offspring when mated with either unrelated wild broodstock or with sibling diploids (Sellars et al. 2012a). Despite these positive results, no current method can induce triploidy in the entire progeny of any given spawning reliably or on a commercial scale. This is also true for other penaeid shrimp species in which triploid induction has been successful and progeny are reproductively sterile; namely, *Marsupenaeus japonicus* (Norris et al. 2005; Sellars et al. 2006b; Coman et al. 2008), *Fenneropenaeus chinensis* (Li et al. 2003c; Li et al. 2006) and *Litopenaeus vannamei* (Xiang et al. 2006).

One possible method of creating all triploid progeny is to create tetraploids and cross them with diploids, to produce all triploid offspring. Tetraploidy has been induced in *F. chinensis* (Li et al. 2003a) and *M. japonicus* (Sellars et al. 2006a) using a heat-shock method; however, no tetraploid embryos have produced viable postlarvae. As a result, this study investigated an alternative tetraploid induction method involving cold shock to determine if tetraploidy can be induced in *P. monodon*.

2.2. Materials & Methods

Wild *P. monodon* broodstock were eyestalk ablated and isolated in spawning tanks set at 27°C. Spawning alarms were used to detect the commencement of a spawning event and marked the zero time post-spawning detection (psd) (Coman et al. 2003). Several cold shock treatments were performed for each spawning event. These shocks were applied to several aliquots of eggs with various parameters for each individual spawning, using methods similar to those applied by Wood et al. (2011). Treatments were applied by pouring eggs and warm spawning tank seawater into 425 ml containers with specific volumes of cold seawater to reach the desired treatment temperatures, which ranged from -2°C to 14°C. In each separate treatment container, this process was performed for 6 min, 8 min, 10 min, 12 min, and 14 min durations. To successfully inhibit first mitosis and induce tetraploids, it is essential to apply the treatment at a precise stage of embryogenesis. However, the spawning detection system is only accurate within a few minutes and may vary depending on the size and duration of an individual spawning. Therefore, the three treatment start times: 18, 20 and 22 min psd, were performed in each treatment to maximise the chances of treating the eggs at the optimal stage of embryogenesis. The cold water treatment was terminated by returning the eggs to 27°C water so cytokinesis could continue; 100 ml of treatment eggs and seawater were sub-sampled into 300 ml of warm seawater (27°C). Developing eggs were then maintained at approximately 27^{o}C until sampling 20 to 24 h psd.

The ploidy status of hatched nauplii (when available) and unhatched (non-viable) eggs in 66 treatments from 10 individual families was determined by taking one sample of 50 unhatched eggs and another of 50 hatched nauplii (when available) approximately 20 h to 24 h psd. Samples were frozen in liquid nitrogen and either processed immediately or stored at -80^{o}C for up to 5 d. Ploidy of groups of 50 eggs/nauplii were assessed using a Cell Lab Quanta SC MPL (Brea, CA, USA) flow cytometer and the Quanta Analysis software (Beckman Coulter Australia Pty Ltd) software as previously described (Foote et al. 2010).

Hatch rates were estimated at 20 to 24 h psd (hatching occurring at approximately 12-14 h) with the aid of a stereo-dissecting microscope. In spawnings/families 1 to 6, estimates of hatch rates were taken from approximately 200 hatched and unhatched (non-viable) eggs (with a minimum 40 hatched nauplii counted and sampled per treatment). In spawnings 7 to 10, all (approximately 500-800) hatched and unhatched eggs were counted.

2.3. Results

Of the 66 treatments, 58 failed to alter the normal diploid state of the eggs, with no treatments above 1°C inducing tetraploidy and no treatments above 5°C inducing polyploids. In total, four of 13 treatments with shock temperatures between -2°C and 1°C induced some tetraploidy, with rates ranging from 14.9% to 24.6%. In addition, four of 14 treatments with temperatures between 2°C and 5°C showed polyploidy with either a mix of diploid, triploid, tetraploids and/or a proportion of mosaicism (Table 1). The proportion of mosaics (if present at all) was unable to be determined as the flow cytometry output was from a group of 50 eggs, not individuals (to obtain a sufficient quantity of DNA for analysis).

Marked reductions in hatch rates were recorded in all treatments relative to controls that were consistently >70% (data not shown). No nauplii hatched in any of the eight treatments that produced polyploids. Furthermore, no nauplii hatched in the 21 treatments from five families treated at between -2°C and 2°C. Hatch rates in the six treatments from two families treated between 3°C and 5°C were extremely low, at less than 0.05%. Nauplii hatched from all 39 treatments from six families treated at 7°C or above, with hatch rates estimated to be greater than 15%, with at least 50 nauplii from the 500 to 800 eggs counted and ploidy analysed (Table 1).

Table 1 Tetraploid *P. monodon* induction rates and nauplii hatch following a range of cold shock treatments

Treatment temperature (℃)	Treatment duration (min)	Family number	Treatment number	Polyploid status (%)	Nauplii hatch
-2	10	7	42[b]	17.4 (4N)	No
-1	8	10	54[c], 60[a]	0	No
	10	7	43[a]	12.9 (4N)	No
		10	55[c], 61[a]	0	No
	12	10	56[c], 62[a]	0	No
0	8	10	57[c]	0	No
	10	7	44[a]	24.6 (4N)	No
		10	58[c]	0	No
	12	10	59[c]	0	No
1	10	7	41[b]	14.9 (4N)	No
2	8	10	63[a]	0	No
	10	6, 9	37[c],40[b], 49[c], 50[c], 64[a]	0	No
		8	47[a]	mosaics[d]	No
	12	10	65[a]	0	No
3	10	8	45[c]	mosaics[d]	No
		9	51[a]	0	Yes[e]
	12	9	52[a]	0	Yes[e]
	14	9	53[a]	0	Yes[e]
4	10	8	46[c]	mosaics[d]	No
5	10	8	48[a]	mosaics[d]	No
7	6	4	19[c]	0	Yes[f]
	10	4, 6	20[c], 39[b]	0	Yes[f]
8	6	4	25[b]	0	Yes[f]
	10	4, 5	26[b], 31[c], 34[b]	0	Yes[f]
9	6	1, 3, 4	5[a], 13[b], 21[c]	0	Yes[f]
	10	1, 3, 4, 5, 6	6[a], 14[b], 22[c], 32[c], 35[b], 38[c]	0	Yes[f]
	6	3	13	0	Yes[f]
10	6	1, 2, 4	1[a], 7[a], 27[b]	0	Yes[f]
	10	1, 2, 4	2[a] 8[a] 28[b]	0	Yes[f]
11	6	1, 2, 3, 4	3[a] 9[a] 15[b], 17[b], 23[c]	0	Yes[f]
	10	1, 2, 3, 4	4[a] 10[a] 16[b], 18[b], 24[c]	0	Yes[f]
12	6	4	29[b]	0	Yes[f]
	10	4, 5	30[b], 33[c], 36[b]	0	Yes[f]
14	6	2	11[a]	0	Yes[f]
	10	2	12[a]	0	Yes[f]

[a]22 min psd treatment
[b]20 min psd treatment
[c]18 min psd treatment
[d]Percent mosaic not determined as ploidy of each individual embryo could not be determined
[e]<0.05%
[f]15 - 50% estimated

2.4. Discussion

This study is the first published instance in which tetraploidy has been induced in *Penaeus monodon*. However, consistent with heat shock induction attempts in other penaeid species, no tetraploid embryos hatched into viable nauplii. It appears that while tetraploidy can be induced at temperatures near-freezing, these low temperatures result in the mortality of all the diploids and tetraploids before they can hatch. It is plausible that the cold shock itself is causing the mortality, as all diploids and tetraploids from each treatment and family died when subjected to the very low temperatures of -2°C to 1°C that appear to be required to induce tetraploids. Cold temperatures had a detrimental effect on survival up until 7°C, with all hatch rates below this temperature less than 0.05%. This study indicates that further investigation of cold shock to inhibit first mitosis in *P. monodon* would not be worthwhile.

In contrast, triploid *P. monodon* nauplii have been produced using cold shock (Wood et al. 2011). However, the temperatures required to induce triploids (6.5°C to 13.8°C) were warmer than for tetraploids (-2°C to 1°C). This indicates that inhibition of polar body II requires less thermal stress than inhibiting first mitosis, and this higher temperature appears less detrimental to embryo survival.

The cause of embryo death in tetraploid *M. japonicus*, induced through the heat shock method, has previously been investigated by Foote et al. (2010). Cytological analysis found that a side effect of the heat shock was irregular mitotic spindle formation that resulted in anucleate cells (lacking a cell nucleus), ultimately leading to embryo death.

The results of both heat and cold shocks support the view that thermal shock is an unsuitable method of producing tetraploid penaeid shrimp. In both thermal shock studies, the effect of doubling ploidy on shrimp viability could not be independently examined as side effects from the thermal shocks caused mortality.

Physical shock methods such as hydrostatic pressure have previously overcome the problem of irregular mitotic spindle regeneration, caused by thermal inductions in Rainbow Trout (Zhang et al. 2007). As there are no reports of pressure shock ploidy manipulation trials in shrimp, this alternative induction method might be one avenue worth investigating, to determine if tetraploid shrimp can be produced.

Authors’ Contributions

A.R. Foote was responsible for experimental design, implementation and analysis of experiments and writing this manuscript/chapter. A.T. Wood assisted with experimental treatments as well as manuscript revision. G.C. Mair and M.J. Sellars assisted with conceptual design as well as logistical and editoral support.

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CHAPTER 3

Tetraploid induction of *Marsupenaeus japonicus* though hydrostatic pressure

Abstract

Tetraploidy has been achieved in a number of penaeid shrimp species by inhibiting cytokinesis during the first cell cycle with a heat or cold shock; however, no tetraploids have survived to postlarval stages. Tetraploid death has been attributed to abnormalities resulting from heat shocks, the high stress of cold shocks or inappropriate timing of treatment application. Subsequently, this study used the novel treatment agent of hydrostatic pressure to determine if viable tetraploids could be produced. Additionally, pressure timing was evaluated with pressure treatments applied at various stages during the first cell cycle to prevent cytokinesis. Tetraploids were induced following a range of parameters: pressure levels 24.1-34.5 MPa applied for 5 or 10 min durations, with treatment initiated from 22-32 min post-spawning detection. However, tetraploid nauplii did not hatch with cytological analysis revealing abnormal anucleate cells, detected from the 4-cell stage, resulted in embryo death, as this abnormality was not resolved later in development. These results are consistent with previous attempts to produce of tetraploid shrimp using heat shocks, as anucleate cells were also detected from 4-cell embryos and resulting in embryo death. Therefore, hydrostatic pressure treatments performed in this study did not overcome abnormalities observed in previous studies, despite applying what is believed to be the minimal pressure stress required to induce tetraploidy at various stages during the cell cycle. All studies to date have not been able to assess the effects of an increased number of chromosomes in shrimp independently of embryo abnormalities.

3.1. Introduction

Commercial scale production of triploid shrimp is desired by the shrimp farming industry as a means of genetically protecting improved shrimp lines from unlicensed breeding, as triploid shrimp are reproductively sterile (Preston et al. 2009; Preston et al. 2010; Sellars et al. 2010). Triploidy can also increase the proportion of females in some shrimp species (Sellars et al. 2009); this is desirable as female penaeid shrimp grow faster than males (Hansford and Hewitt 1994). While triploid shrimp have been produced directly though inhibition of polar body I or II formation, current methods are not suitable for commercial adoption for several reasons. First, current induction methods cannot reliably produce 100% triploidy, which is required to guarantee genetic protection. Second, experimental triploid induction methods are difficult to transfer to a large commercial scale and result in decreased hatch rates. Finally, there is an added degree of complexity to set up and perform these inductions for each spawning event and such inductions would require increased levels of skilled labour. These issues are currently preventing commercial adoption of triploidy on shrimp farms.

An indirect method of triploid production has been previously reported in Rainbow Trout (Chourrout et al. 1986) and the Pacific Oyster (Guo et al. 1996; Wang et al. 2002), which first produced tetraploid broodstock and then mated them with diploid broodstock to create triploid progeny. This indirect method of triploid production would be more commercially desirable, potentially avoiding the current limitations to commercial adoption mentioned above, since inductions would only be required to create tetraploid broodstock. Tetraploid induction has been reported in the penaeid shrimps, *Fenneropenaeus chinensis* (Li et al. 2003a), *Fenneropenaeus indicus* (Morelli and Aquacop 2003), *Marsupenaeus japonicus* (Sellars et al. 2006a) and *Penaeus monodon* (de Almeida Aloise et al. 2011; Chapter 2/Foote et al. 2012). However, all attempts to produce tetraploid broodstock have been unsuccessful to date, with no tetraploid shrimp surviving to postlarval stages.

Tetraploid induction techniques initially used heat shock to inhibit first mitosis, doubling the number of chromosomes in *F. chinensis* (Li et al. 2003a) and *M. japonicus* (Sellars et al. 2006a). However, cytological analysis of the effects of heat in tetraploid *M. japonicus* found that heat was causing an abnormal tetrapolar mitotic spindle formation, which combined with an off-centre pronucleus, resulted in a lethal trait of anucleate cells following first mitosis (Foote et al. 2010). Consequently, cold shock was trialled as an alternative treatment agent to induce tetraploidy in *P. monodon* (Chapter 2/Foote et al. 2012). However, production of tetraploid broodstock remained elusive, as the cold temperatures required to induce tetraploidy were lethal to all progeny regardless of ploidy status. Since the commencement of this research project, tetraploid induction has also been reported in *P. monodon* in another study using cold shock (de Almeida Aloise et al. 2011). However, the induction and development of tetraploids in this study was not clear due to the method of ploidy assessment and hatch counts did not separate individuals of different ploidy.

The present study uses hydrostatic pressure as an alternative treatment agent to inhibit first mitosis in *M. japonicus*, as hydrostatic pressure has previously overcome similar problems of abnormal mitotic spindle formations following heat shock in Rainbow Trout, *Oncorhynchus mykiss* (Zhang et al. 2007).

3.2. Materials & Methods

3.2.1 Broodstock and spawning detection

Marsupenaeus japonicus broodstock were sourced from wild stocks off the east coast of Queensland, Australia. The broodstock were maintained under similar conditions to Coman et al (2008), with a ratio of approximately 25 females to 20 males kept in 2000 L sand substrate tanks, maintained at 28±1°C with flow through, filtered seawater. Broodstock were fed to satiation daily on a diet of chopped squid (*Nototodarus* sp.), commercial *M. japonicus* pellet (Lucky Star, Taiwan Hung Kuo Industrial Co.) and green lipped mussels (*Perna* sp). Impregnated females with ripe ovaries (Crocos and Kerr 1983) were unilaterally eye-stalk ablated then kept in individual 80 L spawning tanks. Up to five 1 L glass trays were located at the base of the tank to collect eggs and a mesh screen placed on top of these trays was used to keep the broodstock separated from the trays (Sellars et al. 2006b). A spawning detection system previously described by Coman et al (2003) was fitted to each tank and sounded an alarm shortly after egg release from the female commenced; initiation of the spawning detector alarm marked the zero time post-spawning duration (psd). The accuracy of detecting the commencement of a spawning event varied between individual spawnings depending on the duration and quantity of eggs released. As egg activation occurs upon contact with seawater (Clark and Lynn 1977), the accuracy of spawning detection was evaluated by observing when embryos divided into 2 and 4 cell stages and comparing this to known cell division times (Hudinaga 1941). Rearing of treated progeny for ploidy assessment was terminated if spawning detection was determined to be >3 min late.

3.2.2. Tetraploid inductions

Two to five glass trays, containing a mix of fertilised eggs and unfertilised eggs, all suspended in seawater, were removed from the tank 4 min prior to treatment. At 2 min prior to treatment, the eggs in seawater were transferred to one control and up to four treatment 1 L cylindrical pressure chambers (Aquatic Eco-Systems Inc., FL, USA) (Fig. 1). Due to availability, initial 'range-finding' experiments used only one chamber per family (Table 1), with up to four chambers available in later experiments (Tables 2 & 3). At the required start time, chambers were pressurised manually to their required pressure level in 5 seconds using a shop press fitted with a 20 tonne hydraulic jack (Fig. 1). As some treatments were performed simultaneously, separate hydraulic presses were used for each chamber. Pressure was maintained for the required duration and was then released in <1 second by releasing the hydraulic jack. The contents of each pressure chamber was then poured into individual 10 L containers containing 28±0.5°C seawater, maintained by a temperature controlled room. Gentle aeration was added 1 h psd.

In total, 83 hydrostatic pressure treatments were performed for 33 families/spawnings, each from a different female. First, a range-finding experiment was performed to determine the pressure range where tetraploidy was induced, 15 treatments were performed for 15 families at pressure levels 13.8-62.1 MPa, for a 5, 8 or 10 min duration, applied at 22 min psd, as this time psd has previously been found to successfully inhibit mitosis in *M. japonicus* (Sellars et al. 2006a; Foote et al. 2010) (Table 1). Second, the optimal pressure level was determined by performing four treatments per family (16-19; Table 2) across the pressure levels 17.2 MPa, 20.7 MPa, 24.1 MPa and 27.6 MPa. Finally, a range of treatment durations and application timings were trialled in 52 treatments from 14 families, all with a pressure level of 24.1 MPa (families 20-33; Table 3).

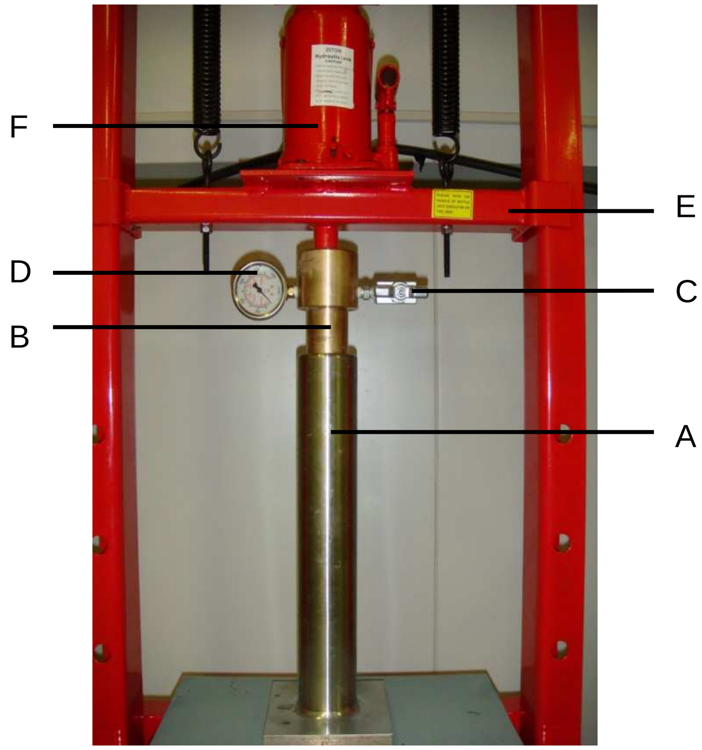


Fig. 1. Pressurisation of *Marsupenaeus japonicus* eggs was achieved with a 1L stainless steel pressure chamber (A) with a brass piston (B), air bleed valve (C) and analogue pressure gauge (D), all rated to 68.9 MPa. Hydrostatic pressure was manually generated using a shop press (E) fitted with an 18 tonne hydraulic jack (F).

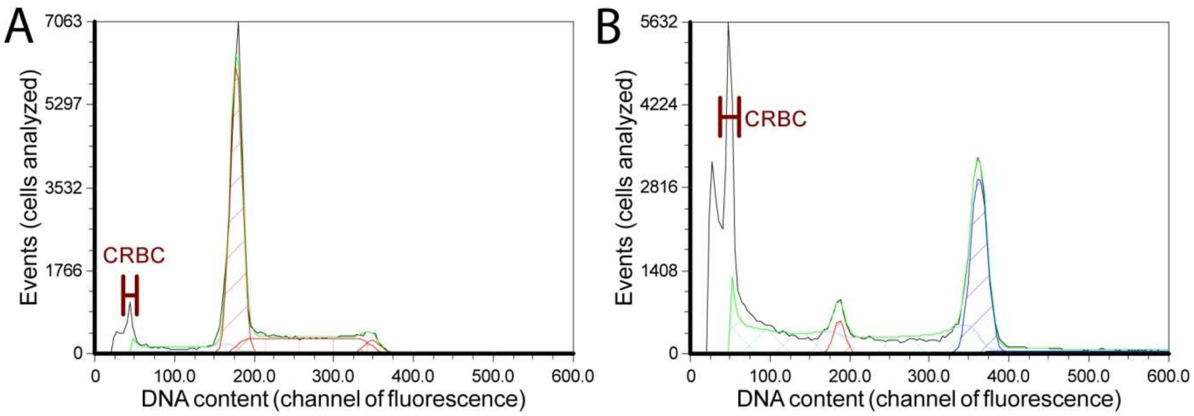
3.2.3. Hatch rate determination

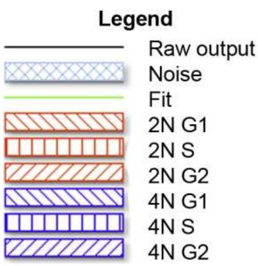
In 'range-finding' experiments (families 1-15; Table 1), the presence or absence of nauplii in the control and treatment was recorded 18-20 h psd. In all other families the hatch rate was calculated 18-20 h psd by counting approximately 300 progeny with the aid of a stereo-dissecting microscope; however, if no nauplii were detected in this initial aliquot all the remaining progeny were counted. To take into account variations in fertility, hatch rates relative to the control (relative hatch rate) were calculated as:

$$\left(\frac{treatment\ hatch\ rate}{control\ hatch\ rate}\right) \times 100$$

3.2.4 Ploidy determination

One hundred 6 h psd embryos and 100 18-20 h psd stage 1 nauplii were sampled, frozen in liquid nitrogen and stored at -80°C for up to one week. To ensure only nauplii were sampled in the 18-20 h psd sample, a stereo-dissecting microscope was used to examine the progeny and exclude any unhatched eggs. The ploidy status was analysed using a Cell Lab Quanta SC MPL (Brea, CA, USA) flow cytometer. This machine was calibrated to maintain low coefficient of variation values within the manufacturer's limits, this was checked on each machine start-up using the manufacturer's quality control fluorescent beads and protocol. The protocol used to analyse shrimp cells was based on that used by Wood et al (2011) and equalled or exceeded the standards outlined by Sellars et al (2010). 350 µL of MPBS propidium iodide stain (MPBS:11.0 g L^{-1}NaCl,0.2 g L^{-1}KCl,1.15 g L^{-1}Na2HPO4 containing 0.1% triton X-100, 0.2mgmL^{-1}Rnase A, 0.02mgmL^{-1}PI) and 7 µL of a1:100 dilution of internal standard glutaraldehyde fixed chicken red blood cells was added. Nauplii were aspirated using a 25G needle and filtered through 63 µm mesh. Sufficient output for accurate ploidy determination was achieved by setting the flow cytometer to count 50,000 shrimp cells. Flow cytometry data was then analysed with the software package FCS express 4 (De-Novo-Software 2012) to determine ploidy.





Cycle	G1 Mean	G1 CV	%G1	G2 Mean	G2 CV	%G2	%S	% total
(A)CRBC	43.54							
(A)Diploid	181.39	4.13	65.11	352.04	2.84	3.88	31.00	100.00
(B)CRBC	47.60							
(B)Diploid	190.59	3.82	100.00	375.47	3.88	0.00	0.00	10.01
(B)Tetraploid	365.91	2.80	83.29	696.28	2.80	2.45	14.25	89.99

Fig. 2. Example of flow cytometry output from an analysis using the FCS express software package (De-novo software 2010). Control (A) contains an internal control of CRBC and diploid *Marsupenaeus japonicus* cells, treatment (B) contains CRBC, diploid and tetraploid *M. japonicus* cells.

3.2.5 Embryonic development

Samples of approximately 100 embryos were taken at 1 h psd and 6 h psd and fixed in either 80% methanol or 70% ethanol for cytological analysis. Following ploidy analysis, three treatment samples with different proportions of tetraploidy as well as control samples from families 22 and 25 were selected for cytological analysis (Table 4). In preparation for cytological analysis, samples were rehydrated by decreasing the concentration of the fixative by 10% at each exchange over 5 h, samples were then washed in PBS 5 times over 3 h to remove any remaining fixative. Samples then incubated in a 1 µg dilution of 4’6-diamidino-2phenylindole (DAPI) in PBS for 3 h. Samples were subsequently washed 5 times with PBS over 3 h and allowed to clear in 70% glycerol in PBS overnight. Samples were mounted on a slide under a cover slip in the glycerol solution for analysis of DNA content using a fluorescent microscope (Zeiss Axiovert 25 with HBO50 illuminator and AxioCam MRc, Carl Zeiss Jena GmbH). *Marsupenaeus japonicus* embryos at 1 h psd were analysed to determine the DNA content in 4-cell stage embryos and 6 h psd embryos were analysed for DNA content and appendage buds formation (Table 4).

3.3. Results

3.3.1. Range-finding tetraploid inductions

Initial hydrostatic pressure trials determined the pressure range between 13.8 MPa and 62.1 MPa that induced tetraploids, as well as the pressure range where nauplii hatched (families 1-15; Table 1). Analysis of variance (one-way ANOVA) found that pressure level had a significant effect on tetraploid induction (10 min duration treatments from Table 1: P=0.03). Flow cytometry analysis of 6 h psd embryos revealed tetraploidy was induced following the pressure treatment levels of 24.1 MPa (family 15), 27.6 MPa (families 6, 12, 14) and 34.5 MPa (family 2) lasting 10 minutes. Tetraploidy was also induced following an 8 minute duration treatment at 34.5 MPa (family 3). Tetraploid induction rates ranged from 26% at 24.1 MPa to 41.2% at 27.6 MPa. All families were fertilised and nauplii hatched from the control aliquot from each. Nauplii hatched following treatment with 13.8 MPa for 5 (family 11) or 10 min (family 10, 13) and 20.7 MPa for a 5 min duration (family 9) but not following a 10 min duration (family 8). No nauplii hatched following pressure levels ≥24.1 MPa.

Table 1: Range-finding hydrostatic pressure induction parameters applied to *Marsupenaeus japonicus* to induce tetraploidy

Pressure (MPa)	Duration (min)	Family	4N embryos (%)(6 h psd)	Hatch (Nauplii detection 24 h psd)	
				Treatment	Control
13.8	5	11	0	yes	yes
	10	10	0	yes	yes
	10	13	0	yes	yes
20.7	5	9	0	yes	yes
	10	8	0	no	yes
24.1	10	15	26.0	no	yes
27.6	5	7	0	no	yes
	10	6	38.3	no	yes
	10	12	27.3	no	yes
	10	14	41.2	no	yes
34.5	5	4	0	no	yes
	8	3	30.2	no	yes
	10	2	22	no	yes
41.4	10	5	0	no	yes
62.1	10	1	0	no	yes

3.3.2. Optimisation of tetraploid induction pressure

Induction parameters were repeated both within and between families by using four treatment pressure chambers to treat four aliquots of eggs in each of families 16-19 (Table 2). Pressure levels of 17.2 MPa, 20.7 MPa, 24.1 MPa and 27.6 MPa for a duration of 10 min were repeated in families 16-18 and in for a 5 min treatment duration in family 19. Consistent with the results from Table 1, one-way ANOVA analysis of treatments performed for a 10 min duration in Table 2 revealed that the treatment pressure level had a significant effect on the induction of tetraploidy ($P=0.004$) and furthermore pressure level had a significant effect on hatch rate ($P=0.02$). Tetraploids were induced in all four families following 24.1 MPa treatments and either a 5 min or 10 min duration, at rates between 6.7% (5 min duration treatment from family 19) and 13.6% (10 minute duration treatment from family 16). Tetraploids were also induced following 27.6 MPa treatments in all but one family (16) with a 10 min duration and at the highest induction rate of 43.5% following a 5 min treatment duration.

As observed in range finding experiments, no nauplii hatched following 10 min duration treatments ≥20.7 MPa or 5 min treatments ≥24.1 MPa. Hatch rates following 10 min duration, 17.2 MPa treatments were greatly reduced with 1.5% to 20.3% of the treatment relative to the control hatching. Anecdotal evidence indicated that hatch rates were higher following pressure treatments performed for a 5 minute duration, with 74.6% hatching following 17.2 MPa and 45.5% hatching following 20.7 MPa.

Table 2: Optimisation of hydrostatic pressure level to induce tetraploidy in *Marsupenaeus japonicus*

Pressure (psi)	Duration (min)	Family	4N embryos (%) (6 h psd)	Hatch rate (%) (24 h psd)	Relative hatch rate[a]
17.2	5	19	0	23.65	74.6
	10	16	0	1.13	1.5
	10	17	0	14.13	18.8
	10	18	0	12.79	20.3
20.7	5	19	0	10.75	45.5
	10	16	0	0	0.00
	10	17	0	0	0.00
	10	18	0	0	0.00
24.1	5	19	6.7	0	0.00
	10	16	13.6	0	0.00
	10	17	11.4	0	0.00
	10	18	12.8	0	0.00
27.6	5	19	43.5	0	0.00
	10	16	0	0	0.00
	10	17	11.5	0	0.00
	10	18	11.3	0	0.00

[a]Relative hatch rate calculated as: $(treatment\ hatch\ rate/control\ hatch\ rate) \times 100$

3.3.3. Optimisation of induction start time and treatment duration

Trials of different treatment application start times and durations, all at a pressure level of 24.1 MPa, revealed that tetraploidy could be induced with a range of parameters (Table 3). Consistent with ANOVA analysis of families 1-15 Tetraploidy was induced in eight families with shocks performed for a 5 min duration at treatment start times of 22 min, 26 min, 28 min, 30 min and 32 min psd. Tetraploids were also induced following the three 10 min duration treatments; one at each of the start times 22 min, 26 min and 32 min psd. The 10 min duration treatment on eggs from family 25, applied at 26 min psd had the highest tetraploid induction rate of 100%. No tetraploids were induced in any of the 17 treatments from eight families performed for the shorter 2½ min duration at any of the start times from 22-40 min psd. There is anecdotal evidence that induction rates from 5 min duration treatments were lower than following 10 min duration treatments; the three families where both a 5 and 10 min duration treatment were performed (20, 22, 25), showed lower induction rates following the 5min duration treatment (Table 3).

No tetraploids were detected at the nauplii stage in any treatment and furthermore, in all the treatments where tetraploidy was induced in a proportion of the embryos, no diploid nauplii hatched either. However, diploid nauplii hatched in some treatments where 0% tetraploidy was induced. In the 25 treatments performed with 5 min shock durations, diploid nauplii hatched in 18 of 15 treatments, with a mean hatch rate relative to their control of 13.2% and a maximum relative hatch rate of 59.3%. The mean hatch rate for the 17 2½ min duration shock duration treatments was 30.6% relative to their control.

Table 3: Optimisation of treatment application timing and duration to induce tetraploid *Marsupenaeus japonicus* through hydrostatic pressure at 24.1 MPa

Treatment start time (min psd)	Treatment duration (min)	Family	4N embryos (%)(6 h psd)	Relative hatch rate (%)[a]
22	2½	20	0	8.9
		21	0	15.5
		23	0	0.6
	5	20	0	5.9
		21	0	7.9
		23	0	0.7
		28	12.7	0.0
	10	20	18.5	0.0
		21	11.3	0.0
		23	13. 9	0.0
24	5	28	0	9.3
		30	0	13.4
		31	0	54.9
		32	0	0.0
		33	0	0.0
26	2½	20	0	55.7
		21	0	51.6
		23	0	70.3
	5	28	16.8	0.0
		29	37.3	0.0
		30	0	19.7
		31	0	59.3
		32	0	0.0
		33	0	41.1
	10	25	100	0.0
28	5	25	31. 8	0.0
		28	15.2	0.0
		29	11.3	0.0
		30	0	0.7
		31	0	0.8
		32	0	0.0
		33	0	0.0
30	2½	25	0	3.0
	5	29	6.5	0.0
		30	0	51.8
		31	0	16.0
		32	0	4.7

		33	0	17.4
32	2½	22	0	49.2
	5	22	0	14.3
		29	15.9	0.0
	10	22	52.4	0.0
34	2½	24	0	14.7
		26	0	15.8
		27	0	7.3
	5	26	0	5.1
		27	0	0.0
36	2½	24	0	1.6
		26	0	0.8
		27	0	0.0
	5	26	0	5.8
		27	0	0.0
38	2½	22	0	78.7
	2½	24	0	76.9
40	2½	24	0	69.8

[a]Relative hatch rate calculated as: $(treatment\ hatch\ rate/control\ hatch\ rate) \times 100$

3.3.4 Embryonic development

Fluorescent microscopic analysis was performed on 50 embryos for each of the three treatments containing different proportions of tetraploids (100%, 52.4% and 31.8%) and the corresponding two controls (from families 22 and 25) (Table 4). Analysis of DAPI stained embryos revealed abnormalities in treatment aliquots containing tetraploids, when compared to their corresponding controls at the 4-cell stage (1 h psd) and at 6 h psd.

Analysis of DNA content in 50 embryos from each of the controls and treatments at the 4-cell stage (1 h psd) revealed two phenotypes. First, embryos with DNA in all cells (Fig. 3A); this was observed in all 100 4-cell stage control embryos and a proportion of the embryos from treatments containing a mix of diploids and tetraploids (Table 4). Second, embryos with DNA in two cells and anucleate cells in the other two cells (Fig. 3B); this was observed in all 50 of the embryos from the treatment containing 100% tetraploids and a proportion of the other two treatments that contained a mix of diploids and tetraploids (Table 4).

Analysis of DNA content and appendage buds in 50 embryos from each of the controls and treatments at 6 h psd, revealed three phenotypes. First, embryos with DNA in all cells as well as six appendage buds (Fig. 3C); this was observed in all 100 control embryos and a proportion of the embryos from the treatments that contained a mix of diploids and tetraploids. The second and third phenotype was embryos with anucleate cells and less than six appendage buds (Fig. 3D, E); this was observed in all 50 embryos from treatments containing 100% tetraploids and proportion of embryos contacting both diploids and tetraploids. Finally, one treatment embryo (31.8% tetraploid) was observed with DNA in all cells; however no appendage buds had formed.

Table 4: The development of 50 control and 50 treatment DAPI stained *Marsupenaeus japonicus* embryos was performed at 1 h psd (4-cell stage) and another 50 control and 50 treatment embryos were analysed at 6 h psd. Embryos were analysed for the presence or absence of a cell nucleus (nucleate/anucleate cells) at both 1 h and 6 h psd, with the presence or absence of appendage buds was also counted 6 h psd.

Family	4N (%)	1 h psd 4-cell embryos with anucleate cells (%) (n=50)	Embryos with anucleate cells (%) 6 h psd (n=50)	Embryos with <6 appendage buds (%) 6 h psd (n=50)
22	0 (control)	0	0	0
	52.4	76	82	82
25	0 (control)	0	0	0
	100	100	100	100
	31.8	60	64	66

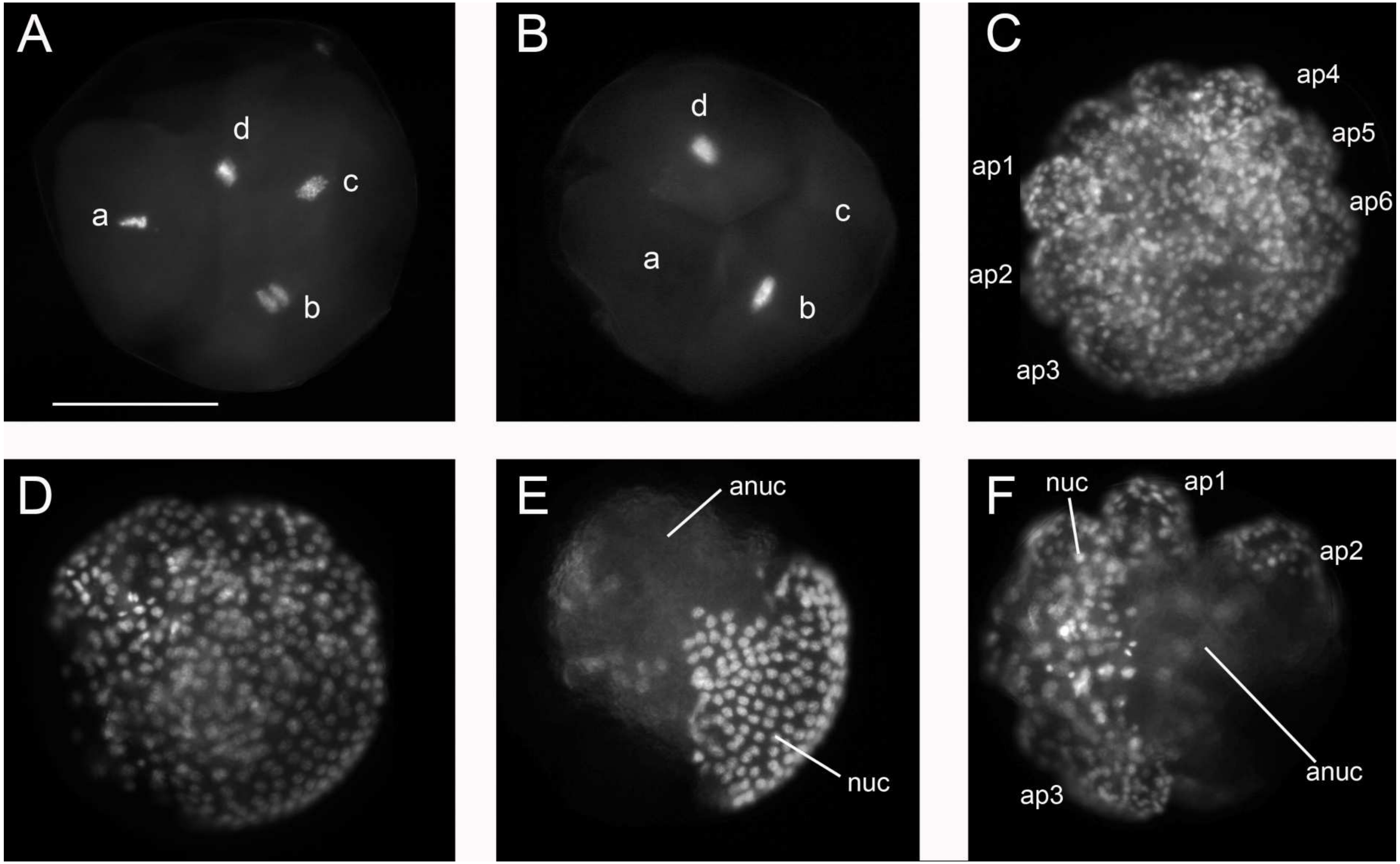


Fig. 3. Fluorescent microscopy images of DAPI stained *Marsupenaeus japonicus* embryos (A-F). Control embryo at 1 h psd, all 4 cells (a-d) containing a nucleus. Treatment embryo at 1 h psd, 2 cells (b, d) containing a nucleus, while 2 cells (a, c) are anucleate. A control embryo 6 h psd with 6 appendage buds (ap1-6). Treatment embryos 6 h psd (D-F) with no appendage bud formation (D, E), some resemblance of three appendage buds (ap1-3) and some cells with (nuc) and without a nucleus (anuc). Scale bar = 100µm.

3.4. Discussion

3.4.1. Tetraploid induction & viability

Hydrostatic pressure was used as a novel treatment agent to induce tetraploidy in *Marsupenaeus japonicus* embryos. Despite trialling an extensive range of induction parameters, production of viable tetraploid larvae was not achieved as all tetraploids died during embryogenesis. The induction of non-viable tetraploids is consistent with all previous reports in penaeid shrimp, which all used thermal shocks: *Fenneropenaeus chinensis* (Li et al. 2003a), *Fenneropenaeus indicus* (Morelli and Aquacop 2003), *M. japonicus* (Sellars et al. 2006a; Foote et al. 2010) and *Penaeus monodon* (de Almedia Aloise et al. 2011; Foote et al. 2012/Chapter 2).

The critical pressure level capable of producing viable tetraploid progeny needs to be both strong enough to induce tetraploidy, but not to strong that it is lethal to all progeny. In the previous study that used cold shock to induce tetraploid *P. monodon* (Foote et al. 2012/Chapter 2) this critical level was not achieved as the cold temperature required to induce tetraploidy was lethal to all progeny. In the current study, a 5 min pressure treatment at 24.1 MPa was the minimum pressure level that induced tetraploid *M. japonicus* and also the maximum pressure level at which some diploid progeny could survive and hatch. However, in each treatment where tetraploidy was induced to a proportion of the progeny, no diploids hatched. It is unclear why diploids never hatched in treatments where a proportion of tetraploids had been induced, despite the minimum pressure level required to induce tetraploidy demonstrated to be survivable, as diploids hatched on some occasions following this pressure level.

A greater level of hydrostatic pressure was required in the current study to delay cytokinesis in *M. japonicus* and induce tetraploidy than was required to inhibit polar body II formation and induce triploidy in *M. japonicus* (Chapter 4). Triploid *M. japonicus* were achieved following hydrostatic pressure at levels of 6.9-34.5 MPa applied 7½ min psd for a 10 min duration (Chapter 4), compared to pressure levels 24.1-34.5 MPa in the current study, applied ≥22 min psd for various durations. In both studies no nauplii (including diploids) hatched following treatment with ≥24.1 MPa for 10 minutes.

By comparing these studies on *M. japonicus* ploidy manipulation, it could be concluded that greater amounts of pressure shock are required to depolymerise mitotic spindle formations and prevent cytokinesis at the end of the first cell cycle than is required to inhibit polar body II formation during meiosis. The requirement of greater shock/stress levels to induce tetraploidy compared to triploidy is consistent with the findings of previous studies that used cold shock to induce both triploidy and tetraploidy. Cold shocks at 6.5°C to 13.8°C were able to produce triploid *P. monodon* (Wood et al. 2011), while cold shocks at 1°C to -2°C were required to induce tetraploidy in *P. monodon* (Foote et al. 2012). It can therefore be concluded that a greater amount of pressure shock is required to depolymerise mitotic spindle formations and prevent cytokinesis at the end of the first cell cycle than is required to inhibit polar body II formation during meiosis.

3.4.2. Tetraploid abnormalities

Cytological analysis of a total of 150 embryos from three treatments and a total of 100 embryos from two controls, identified abnormalities exclusive to treatment embryos. An abnormal trait of anucleate cells was detected from the 4-cell stage and was not resolved later in embryogenesis as the lack of a cellular checkpoint for abnormal spindle architecture allows anucleate cells to continue dividing (Sluder et al. 1997). Since this abnormality was not resolved, normal cell differentiation into nauplii did not occur and all tetraploids died before reaching the nauplii stage.

The anucleate cell phenomenon has been previously observed following heat shocks timed to suppress mitosis in *F. indicus* (Morelli and Aquacop 2003) and *M. japonicus* (Foote et al. 2010). In these earlier studies, normal pronuclear migration towards the cell cortex (Hertzler and Clark 1993) failed to complete, with an abnormal off-centre pronucleus and/or an abnormal mitotic spindle assembly observed and determined to be due to the heat shock disrupting the microtubules which are required for such migrations (Hertzler and Clark 1993). These studies suggested that abnormal DNA orientation and abnormal cell division could then lead to anucleate cells (Morelli and Aquacop 2003; Foote et al. 2010). In the case of *M. japonicus*, a tetrapolar mitotic spindle formation and off-centre pronucleus during the second cell cycle was the only phenotype observed in treatments which resulted in tetraploid embryos (Foote et al. 2010). The anucleate cells observed in the current pressure treated embryos appears to be consistent with the findings from heat shocked tetraploid *M. japonicus* embryos (Foote et al. 2010).

Observations of abnormal mitotic spindle architecture and abnormal cell cleavage has not been limited to penaeid shrimp, with abnormal tripolar and tetrapolar spindle formations observed in some embryos following mitotic heat shocks in Rainbow Trout, *Oncorhynchus mykiss*, which resulted in cleavage from one cell into three or four cell embryos (Zhang et al. 2007). However, inconsistent with *M. japonicus* (Foote et al. 2010) no off-centre pronucleus or anucleate cells were observed. Abnormal spindle architecture was however avoided in *O. mkyiss* by using the alternative treatment agent of hydrostatic pressure to inhibit cleavage (Zhang et al. 2007), a result inconsistent with the current study, where cytological abnormalities persisted in *M. japonicus* following hydrostatic pressure treatment.

It has be suggested that abnormal spindle formation and an off-centre pronucleus may be overcome by applying the treatment at alternative stages of the cell cycle (Morelli and Aquacop 2003). However, in the current study no viable tetraploid nauplii were produced despite applying treatments at a range of times during the cell cycle (22-40 min psd) and for various durations (2½ min, 5 min and 10 min).

3.4.3 Conclusion

Hydrostatic pressure was used to successfully induce tetraploid *M. japonicus* embryos for the first time. However, tetraploid embryos were not viable and did not reach the nauplii stage due to lethal cellular abnormalities consistent with previously reported abnormalities in other tetraploid shrimp studies that used heat shock induction agents (Morelli and Aquacop 2003; Foote et al. 2010). The minimum pressure shock to induce tetraploidy was 5 min at 24.1 MPa, further work on tetraploid induction may benefit from using these parameters as a starting point when investigating the viability of tetraploidy in different penaeid species.

Authors' Contributions

A.R. Foote was responsible for experimental design, implementation and analysis of experiments and writing this chapter. G.C. Mair and M.J. Sellars assisted with conceptual design as well as logistical and editoral support.

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CHAPTER 4

Triploid *Marsupenaeus japonicus* production using hydrostatic pressure

A version of the following chapter was submitted to the journal *Aquaculture* as:

Foote. A.R, Mair. G.C and Sellars. M.S. Triploid *Penaeus japonicus* (Kuruma Shrimp) production using hydrostatic pressure. Aquaculture (submitted).

Abstract

While triploidy has been induced in a number of shrimp species, there are no effective methods for commercial production due to difficulties in treating an entire spawn without physically damaging developing embryos. By spawning shrimp inside a large hydrostatic pressure chamber, which is then used to apply a pressure shock to induce triploidy, an entire spawn may be treated without the need to handle embryos. The current study has demonstrated that hydrostatic pressure can be used to inhibit meiosis II in *Marsupenaeus japonicus* (Kuruma Shrimp) to produce viable triploids. This is the first known report in Crustacea demonstrating that hydrostatic pressure can be used to manipulate ploidy. In this study the pressure range required to induce triploidy was optimised with small experimental sized pressure chambers. Triploid stage 1 nauplii were produced following pressure treatments of 13.8-20.7 MPa for a 10 min duration, 7 ½ min post-spawning detection. The highest proportion of triploids was produced at 17.2 MPa, which was also the most efficient pressure trialled and 100% triploid nauplii was achieved in three of seven families. Future studies may transfer this technique to a large hydrostatic pressure chamber that can accommodate a whole spawning and also to other commercially farmed species of shrimp in order to determine if production of triploids using hydrostatic pressure is commercially viable.

4.1. Introduction

Selective breeding programs for commercially important shrimp species have resulted in the production of genetically superior lines, which grow faster and have improved survival. These advances have improved the reliability and efficiency of shrimp farming, resulting in high value shrimp lines which need to be genetically protected before the seedstock can be sold on to other farms to grow-out (Preston et al. 2004; Coman et al. 2006). Genetic protection will ensure the economic investments associated with selective breeding programs are protected. Several methods of genetically protecting shrimp through reproductive sterilisation have been investigated, including ionizing irradiation (Sellars et al. 2005) and gene regulation (Sellars and Preston 2008; Callaghan et al. 2010). However, triploidy is the only current method known to successfully produce reproductively sterile shrimp, guaranteeing genetic protection.

Triploid shrimp can be induced by inhibiting polar body I or II extrusion during meiosis. This has been achieved in a number of commercially farmed penaeid shrimp species globally (Reviewed by Sellars et al. 2010), with triploids reared to adult age in Chinese Shrimp, *Fenneropenaeus chinensis* (Li et al. 2003c); Kuruma Shrimp, *Marsupenaeus japonicus* (Norris et al. 2005); and Black Tiger Shrimp, *Penaeus monodon* (Sellars et al. 2012b). Reproductive sterility of triploid penaeids has been demonstrated through analysis of reproductive tissues in *F. chinensis* (Li et al. 2003b; Xiang et al. 2006) and *M. japonicus* (Sellars et al. 2009). Reproductive performance trials in *P. monodon* have also demonstrated that triploids are reproductively sterile, even when crossed with diploids (Sellars et al. 2012b).

In addition to the benefit of genetic protection, *M. japonicus* triploids produced through inhibition of polar body II are always female (Sellars et al. 2009). As female shrimp grow faster than males (Hansford and Hewitt 1994; CSIRO unpub.) farming production could be improved by stocking ponds with a greater proportion of females.

A range of treatment methods have been trialled to induce triploidy in shrimp (Sellars et al. 2010), with the chemical 6-dimethylaminopurine (6-DMAP) (Sellars et al. 2006b) and heat shocks (Li et al. 2003c) most successful. While triploids have been successfully induced at an experimental scale using these treatment methods, they are not suitable on a commercial scale as it is difficult to apply and/or remove a treatment effectively for an entire spawn. Current triploid induction techniques involve handling embryos at early, fragile stages of development to apply and/or remove the treatment, resulting in reduced hatch rates due to handling stress.

Hydrostatic pressure is another treatment method that is yet to be trialled on penaeid shrimp, but is proven successful for other aquatic species including salmon and oysters. Triploids are farmed commercially in species such as salmon and oysters (Nell 2002) to improve farming efficiency and increase product value. The hydrostatic pressure technique could overcome the current problems hindering commercial production of triploid shrimp, by spawning the shrimp in a large hydrostatic pressure chamber. This would ensure the fragile embryos are not handled and allow treatment of an entire spawning.

This study reports on triploid *M. japonicus* production by hydrostatic pressure treatment for the first time and includes triploid induction optimisation parameters for this species. If successful, such techniques may be scaled up to a spawning tank sized pressure chamber and ultimately, the technique may be transferred to more commercially important species such as *P. monodon*.

4.2. Materials and Methods

Mature wild-caught *Marsupenaeus japonicus* broodstock were caught off the east coast of Queensland, Australia, maintained in two tonne seawater tanks at 27±1°C and fed on a maturation diet as described by Coman et al. (2008). Thirty-two females with stage IV ovaries (Crocos and Kerr 1983) were unilaterally eyestalk ablated (Norris et al. 2005). Two days later the shrimp were isolated in individual 80 L spawning tanks with seawater at 27°C. Spawning alarms detected the commencement of egg release (Coman et al. 2003) and marked the zero time post-spawning detection (psd).

In total, there were 32 spawning events, of which 20 were detected within three minutes of spawning, as determined by the known two and four cell division times psd (Hudinaga 1941). From these 20 spawnings, up to four treatments were performed per spawning, giving a total of 41 treatments (Table 1), which were assessed for hatch rate and ploidy level as described below.

Two to five 1 L glass trays/aliquots of eggs were removed from the spawning tank 5 min psd. The contents of one tray of eggs was gently poured into a 1 L cylinder at 6 min to 6 ½ min psd to mimic handling stress and used as the control. The contents of each of the remaining glass trays were gently poured into separate 1 L pressure chambers at 6 ½ min to 7 min psd. Pressure chambers were constructed of stainless steel and had a brass piston with an air bleed valve and pressure gauge rated to 68.9 MPa (Aquatic Eco-Systems Inc., FL, USA). Air was bled from the chambers and an 18 tonne hydraulic press was used to manually pressurise the vessels to their required treatment level, within 5 sec, at 7 ½ min psd. Pressure was maintained at the desired level for a 10 min duration, then released instantly by opening the air bleed valve. All treatment and control eggs in seawater were then gently poured into separate 10 L containers and maintained at 27°C, with gentle aeration for 24 h until hatching.

Hatch rates for each control and treatment were determined 24 h psd (approximately 10 to 12 h following hatch) by counting 300 progeny under a stereo dissecting microscope. The treatment hatch rate relative to the control hatch rate (relative hatch rate) was then calculated as:

$$\left(\frac{treatment\ hatch\ rate}{control\ hatch\ rate}\right) \times 100$$

'Triploid production efficiency' was defined as the proportion of triploid nauplii produced from the total number of viable eggs (the control hatch rate) and calculated as:

$$\left(Treatment\ triploid\ nauplii\ percent \times \left[\frac{treatment\ hatch\ rate}{control\ hatch\ rate}\right]\right) \times 0.01$$

One hundred embryos and 100 nauplii per control and treatment were separated with the aid of a stereo dissecting microscope 24 h psd and frozen in liquid nitrogen for ploidy analysis using a Cell Lab Quanta SC MPL (Brea, CA, USA) flow cytometer. An internal control of chicken red blood cells was included in each sample to ensure there was no spectral shift in the diploid and triploid peaks (Sellars et al. 2006a). Each output was analysed using the 'multi-cycle' function in the software program *FCS express 3,* (De-Novo-Software 2011) to determine ploidy status. Triploid rates were recorded when readings in this region were greater than 5%.

Table 1: Experimental design for the 41 pressure treatments from 20 families of *Marsupenaeus japonicus*

Family	Treatment pressure (MPa)			
	Aliquot 1	Aliquot 2	Aliquot 3	Aliquot 4
1	34.5[a]			
2	27.6[a]			
3	20.7			
4	13.8[a]			
5	13.8[a]			
6	13.8			
7	10.3			
8	6.9			
9	17.2			
10	13.8	17.2		
11	10.3	13.8		
12	13.8	17.2	20.7	
13	13.8	17.2	20.7	27.6[b]
14	13.8	20.7		
15	3.4[c]	6.9[c]	10.3[c]	13.8[c]
16	6.9	20.7	24.1[a]	
17	6.9	17.2		
18	17.2[c]	24.1[a]		
19	10.3	13.8	20.7	24.1[b]
20	10.3	13.8	17.2	20.7

[a] No hatch rate data
[b] No nauplii data as no nauplii hatched
[c] No embryo data due insufficient DNA content (presumably from a high proportion of infertile eggs or non-viable embryos in the sample)

4.3. Results

Of the 41 treatments from 20 families analysed, triploidy was detected in embryos from 30 treatments and 24 h psd triploid stage 1 nauplii in 20 treatments (Table 2). Analysis of variance (one-way ANOVA) indicated that pressure level had a significant effect on triploid induction (P=<0.001), while the family effect on triploid rate was also significant (P=0.0151). Triploid induction rates at 20.7 MPa and 24.1 MPa had the highest mean induction rates of 63.7% and 55.8% respectively (Fig. 1A), with triploid induction rates at these two pressure levels significantly different (LSMEANS) to all other pressure levels except 17.2 MPa. Rates of 100% triploid embryos were achieved in two treatments (families 18 & 20) following treatment at 20.7 MPa and 24.1 MPa (Table 2); no embryos hatched in these treatments (Table 2).

Triploid nauplii were produced on 20 occasions, following pressure treatment at 13.8, 17.2 or 20.7 MPa (Table 1). Analysis of variance (one-way ANOVA) indicated that pressure level had a significant effect on triploid induction rate (P=0.001), while the family effect was not significant (P=0.2712). The maximum mean triploid rate at the nauplii stage was 77.6% at 17.2 MPa (Fig. 1B), which was significantly different (LSMEANS) to all other pressure levels. Triploid nauplii induction rates of 100% were achieved on three of eight occasions at this pressure (Table 1).

Nauplii (diploid and/or triploid) hatched following pressure shock across the range 3.4-20.7 MPa (Table 1). Pressure level had a significant effect on hatch rate (one-way ANOVA), with the highest mean hatch rate achieved following shocks at 6.9 MPa (Fig. 1C), a level which had a significantly different hatch rate to all other pressure levels (LSMEANS). The family effect on hatch rate relative to controls was not significant (P=0.9429: one-way ANOVA). Mean hatch rates across the pressure range which produced triploid nauplii (10.3-20.7 MPa), showed a spike at 17.2 MPa with 46.6% nauplii hatch, this dropped to 37.4% and 27.3% hatch at 13.8 and 20.7 MPa respectively (Fig 1C). Three treatments (families 12, 13 & 18) with 100% triploid nauplii were achieved, all following pressure treatments at 17.2 MPa (Table 2). While all treatments performed at pressures greater than 20.7 MPa resulted in no hatched nauplii (Table 1; Fig. 1C).

Analysis of triploid production efficiency also showed pressure level had a significant effect (one-way ANOVA). The pressure shock level of 17.2 MPa was optimal, with the highest mean of 54.7% achieved, significantly different to all other pressure levels (LSMEANS). The family effect did not have a significant effect on production efficiency. Within family analysis of the three families each with treatments performed at 13.8, 17.2 and 20.7 MPa (families 12, 13, 20), shows triploid production rate and production efficiency were greatest at 17.2 MPa (Table 1).

Table 2. Forty-one hydrostatic pressure inductions from 20 families of *Marsupenaeus japonicus*, aimed at inhibiting polar body II formation and induce triploidy. Triploid rate for embryos and nauplii 24 h psd, as well as treatment hatch rate relative to their control and production efficiency[b]

Pressure (MPa)	Family	3N embryo (%)	3N nauplii (%)	Hatch rate relative to control (%)[a]	Production efficiency (%)[b]
8	15	*	0.0	34.7	0.0
6.9	8	23.7	0.0	103.9	0.0
6.9	15	*	0.0	27.5	0.0
6.9	16	0.0	0.0	125.0	0.0
6.9	17	12.9	0.0	148.6	0.0
10.3	7	0.0	0.0	0.0	0.0
10.3	11	0.0	0.0	69.9	0.0
10.3	15	*	0.0	27.5	0.0
10.3	19	0.0	0.0	12.8	0.0
10.3	20	0.0	0.0	35.3	0.0
13.8	4	75.7	75.1	hatch*	-
13.8	5	27.7	7.0	hatch*	-
13.8	6	31.8	20.1	32.3	6.5
13.8	10	22.3	29.9	64.0	19.1
13.8	11	30.2	37.3	52.9	19.7
13.8	12	37.0	54.9	64.1	35.2
13.8	13	7.5	0.0	0.0	0.0
13.8	14	13.7	10.4	43.8	4.5
13.8	15	*	41.4	1.5	0.6
13.8	19	0.0	0.0	53.9	0.0
13.8	20	8.2	12.1	35.3	4.3
17.2	9	70.3	82.1	61.0	50.0
17.2	10	71.9	61.6	53.3	32.8
17.2	12	72.6	100.0	70.5	70.5
17.2	13	45.4	100.0	94.6	94.6
17.2	17	18.9	28.3	20.0	5.7
17.2	18	*	100.0	75.8	75.8
17.2	20	42.1	71.0	75.3	53.4
20.7	3	63.1	0.0	0.0	0.0
20.7	12	79.5	85.3	58.7	50.1

20.7	13	81.5	96.6	60.3	58.2
20.7	14	67.7	13.1	11.9	1.6
20.7	16	60.3	0.0	0.0	0.0
20.7	19	12.0	18.4	32.7	6.0
20.7	20	100.0	0.0	0.0	0.0
24.1	16	60.8	-	0.0	0.0
24.1	18	100.0	-	0.0	0.0
24.1	19	51.4	-	0.0	0.0
27.6	13	28.1	-	0.0	0.0
27.6	2	38.8	-	0.0	0.0
34.5	1	35.0	-	0.0	0.0

[a]hatch rate relative to control: $(treatment\ hatch\ rate/control\ hatch\ rate) \times 100$
[b]production efficiency:
$(Treatment\ triploid\ nauplii\ percent \times [treatment\ hatch\ rate/control\ hatch\ rate]\ ) \times 0.01$
*ploidy analysis not possible due to insufficient DNA content

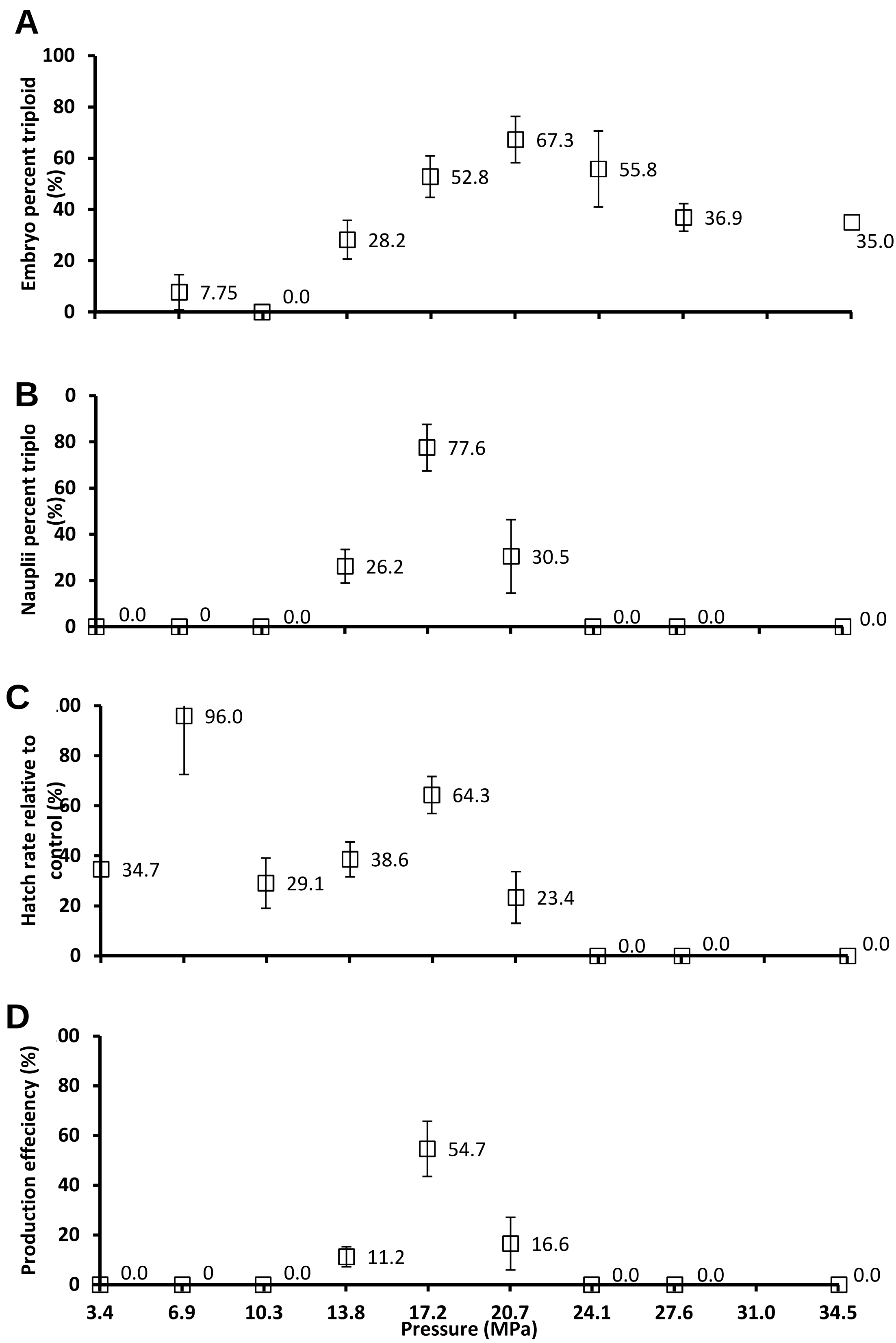

Fig. 1. *Marsupenaeus japonicus* triploid induction rate following hydrostatic pressure shock treatment, detected in 6 h psd embryos (A) and 24 h psd stage 1 nauplii (B). *M. japonicus* hatch rate relative to its control hatch rate following hydrostatic pressure shock treatment $(treatment\ hatch\ rate/control\ hatch\ rate) \times 100$ (C) and the efficiency of triploid production $(Treatment\ triploid\ nauplii\ percent \times [treatment\ hatch\ rate/control\ hatch\ rate]\ ) \times 0.01$ (D)

4.4. Discussion

This study is significant as it is the first instance in which hydrostatic pressure has been used to induce triploidy in a penaeid shrimp species and moreover it is the first known report of polyploid production using hydrostatic pressure in any crustacean. The hydrostatic pressure level required to produce *Marsupenaeus japonicus* triploids was optimised; determining high and low thresholds where triploid nauplii did not hatch, the pressure that produced the greatest proportion of triploids, the most efficient pressure level and the pressure that produced 100% triploidy with the greatest frequency.

The optimal pressure level required to produce triploid nauplii was 17.2 MPa which was significantly different to all other pressure levels; this level of 17.2 MPa resulted in the greatest mean proportion of triploid nauplii at 77.6%. The pressure range required to produce triploids was also determined, with pressure levels at or below10.3 MPa insufficient for triploid nauplii production and pressure levels at or above 24.1 MPa lethal, as this pressure level always resulted in the death of all treated diploids and triploids.

The most efficient pressure level required to induce triploids was also 17.2 MPa. Relative hatch rates were incorporated into the production efficiency formula to determine the proportion of triploids produced relative to the proportion of viable embryos. Calculating the hatch rate of a treatment relative to its control is necessary as there can be great differences in fertility and other factors influencing hatch rates between families and spawnings. For example in family 13, there is little difference in the proportion of triploid nauplii produced following 17.2 MPa and 20.7 MPa (3.4%). However, analysis of the triploid production efficiency with this same family shows a large difference of 34.3% between treatment at 17.2 MPa and 20.7 MPa. Production efficiency would be important in commercial applications to factor in any loss in hatch rate when performing triploid inductions.

One hundred percent triploid nauplii were produced from three treatments at 17.2 MPa. The frequency of 100% triploid inductions will be commercially important if total sterility is desired to guarantee genetic protection of stock sold on to other farms. However, production efficiency may be more important if the commercial goal is to increase production on their own farm, by increasing the proportion of females which grow faster than males (Hansford and Hewitt 1994). Production of females would be of particular interest in *M. japonicus*, where all triploids induced through inhibition of polar body II are female (Sellars et al. 2009).

Triploidy was also detected in unhatched embryos; however, the pressure range where triploid embryos were detected (6.9-34.5 MPa) was different to the pressure range where triploid nauplii hatched (13.8-20.7 MPa). While it is logical that a shock at too high a pressure would result in embryo death and thus no hatched nauplii, it is unclear why triploid embryos detected at low threshold of 6.9 MPa and 13.4 MPa never hatched into nauplii. Further, it is unclear why relative hatch rates were significantly lower following the lower pressure treatment level of 10.3 MPa compared to 17.2 MPa.

Hydrostatic pressure shock is a technique that has been used previously to inhibit meiosis or mitosis in a number of salmonids and other fishes to produce triploids, tetraploids, meiotic gynogens and mitotic gynogens. Triploid salmonids induced through this method include Rainbow Trout, *Salmo gairdneri* (Onozato 1984; Chourrout 1986)*;* Cherry Salmon, *Oncorhynchus masou* (Onozato 1984); Chum Salmon, *Oncorhynchus keta* (Onozato 1984), Atlantic Salmon, *Salmo salar* (Johnstone and Stet 1995) and Coho Salmon, *Oncorhynchus kisutch* (Piferrer et al. 1994). Hydrostatic pressure has also been used to manipulate the ploidy of Channel Catfish, *Ictalurus punctatus* (Goudie et al. 1995), Walleye, *Stizostedion vitreum* (Malison and GarciaAbiado 1996) and European Sea Bass, *Dicentrarchus labrax* (Peruzzi and Chatain 2000). The pressure required to inhibit meiosis or mitosis in these fishes is typically 55.2-68.9 MPa; this is much higher than the 13.8-20.7 MPa range for *M. japonicus* in the current study. The lower pressure level required to induce triploidy in *M. japonicus*, indicates that this species is more fragile during meiosis.

Hydrostatic pressure has also been used to induce triploidy in some molluscs at a pressure range similar to the optimal 17.2 MPa level for *M. japonicus* in the current study. A pressure of 19.6 MPa was used to induce triploid Pacific Abalone, *Haliotis discus hannai* (Arai et al. 1986); Noble Scallop, *Chlamys nobilis* (Komaru and Wada 1989a); and Pearl Oyster, *Pinctada martensii* (19.6 MPA-24.5 MPA) (Shen et al. 1993). While triploid Pacific Oyster, *Crassostrea gigas* have been induced at a higher pressure of 41.4-55.2 MPa (Chaiton and Allen 1985; Allen et al. 1986).

4.4.1 Conclusion

This study has achieved the aim of producing triploidy in shrimp through the hydrostatic pressure technique. The optimal pressure level of 10 minutes of 17.2 MPa and the pressure range of 13.8-20.7 MPa will be useful for any future research with this technique in *M. japonicus* and will provide a valuable starting point when transferring this technique to other penaeid species. However, there is still scope to refine pressure duration and treatment application to further optimise desired induction and production efficiency levels. This technique may now be transferred and trialled in a large spawning tank sized pressure chamber to allow treatment of an entire spawn with reduced handling stress; potentially overcoming the current limitations for commercial production of triploid shrimp. Once triploid production is achievable on a commercial scale, the survival of triploids through a commercial hatchery could then be evaluated. Further, this hydrostatic pressure technique may be transferred to and optimised for other species of shrimp with more commercial interest and the performance of triploids evaluated in a commercial hatchery.

Authors' Contributions

A.R. Foote was responsible for experimental design, implementation and writing this chapter, as well as assisting with broodstock husbandry. M.J. Sellars and G.C. Mair provided conceptual support as well as editorial assistance.

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CHAPTER 5

Hydrostatic pressure progresses triploid shrimp production closer to commercial reality

Abstract

Triploid shrimp are reproductively sterile and as such, triploidy provides an avenue for genetic protection of selectively bred shrimp lines. However, current research based methods of shrimp triploid induction are unsuitable for commercial adoption due to inconsistent triploid induction rates, low hatch rates and the methods are impractical on a large scale. This study makes significant progress in the endeavour to commercialise shrimp triploidy; successfully transferring the hydrostatic pressure technique to the commercial species *Penaeus monodon* and then engineering a novel large pressure chamber (LPC) capable of inducing triploidy to entire spawnings/families of penaeid shrimp without handling the progeny to apply or remove the treatment.

Firstly, the hydrostatic pressure technique was applied to *P. monodon* using small experimental sized pressure chambers (SPCs) to induce triploidy. Optimal induction parameters were determined by using up to four SPCs to perform multiple treatments per family. Highest triploid induction levels were achieved in *P. monodon* following 17.2 MPa and 20.7 MPa, with 100% triploid rates recorded in several families following treatment at these pressure levels. Second, the pressure tolerance of *P. monodon* broodstock was evaluated, with 100% survival following 10 minutes of 17.2 MPa pressure stress. Finally, a prototype LPC was developed, and by using previously published optimal induction parameters for *M. japonicus*; a pressure level of 17.2 MPa, applied from 7-17 min psd, resulted in 100% triploidy in two of three *M. japonicus* families, when applied under optimal treatment conditions.

5.1. Introduction

Genetically improved penaeid shrimp lines have been achieved around the globe in recent years, improving commercial farm production. In Australia, the greatest advances have been achieved in the Black Tiger Shrimp, *Penaeus monodon*, which is the major farmed species in the country. After several generations of selective breeding *P. monodon*, farm harvest yield per hectare has more than doubled when compared to stocks derived from wild caught broodstock (Preston et al. 2010). In addition, selected lines of *P. monodon* have increased survival and feed conversion efficiency (Glencross et al. 2010). Protection of intellectual property and breeders rights related to these improved lines is inhibiting more widespread production of improved lines. Methods of inhibiting reproduction in these improved lines would prevent unlicensed breeding, allowing high value postlarvae to be sold to other farms to grow out to harvest size expanding the benefits of the genetic improvement across the industry.

Triploid induction is the only technique currently known to guarantee inhibition of reproduction in penaeid shrimp and has been studied in several shrimp species (Sellars et al. 2010) including the Chinese Shrimp, *Fenneropenaeus chinensis* (Li et al. 2003c), the Kuruma Shrimp, *Marsupenaeus japonicus* (Norris et al. 2005) and *P. monodon* (Wood et al. 2011). Triploid shrimp have been successfully induced by preventing polar body I or II extrusion during meiosis using various treatment methods including chemical and thermal shocks (Sellars et al. 2010). However, in all species and induction methods, triploid production has not been reliably or consistently achieved at high or absolute rates and additional problems arise when adapting these experimental methods, performed on sub-sets of a spawning, to treat the entire spawning which would be required for commercial adoption. One such problem is the reduction in hatch rates from treated families, as all protocols require handling of the fragile eggs/embryos to apply and/or terminate the treatment. In addition, methods involving handling of progeny would be impractical to perform in a commercial hatchery as it would require a high investment in time from skilled labour.

In this study, treatment with hydrostatic pressure will be investigated due to its success at inducing triploidy in the *M. japonicus*, with 100% induction rates achieved in several families, under optimal conditions (Chapter 3). In addition to the high induction rates achieved with this technique, treatment using a hydrostatic pressure chamber has an advantage over other methods from an engineering perspective, as the size of the pressure chamber can be readily increased to allow shrimp to spawn inside the chamber. By using the pressure chamber as the spawning tank, an entire spawning could be treated without the need to transfer any progeny during embryogenesis. In addition, a large hydrostatic pressure chamber (LPC) could be engineered apply treatments with increased autonomy though the use of electronic, pre-programmed components.

The current study focuses on the commercial potential of producing triploid shrimp through hydrostatic pressure treatment by performing a sequence of experiments to optimise induction protocols. First, this technique is transferred from the protocol and parameters reported in this study for *M. japonicus* (Chapter 3) and optimised for *P. monodon* using four small pressure chambers (SPCs), which permitted multiple treatments to be performed per family. Second, the pressure tolerance of *P. monodon* broodstock is determined to assist in the engineering of a large pressure chamber (LPC) induction method, as female broodstock will remain in the LPC during pressure treatment. Third, a prototype LPC is engineered and triploid induction trials performed on *P. monodon* and *M. japonicus*, to determine if the current limitations towards commercialisation of triploidy can be reduced or eliminated with this method.

5.2. Materials & Methods

5.2.1 Penaeus monodon triploid optimisation using small pressure chambers

Domesticated ninth generation *Penaeus monodon* broodstock (~90 g) from a commercial farm in south-east Queensland, Australia, were maintained under similar conditions to those described by Wood et al (2011), with broodstock held in 10,000 L tanks, receiving flow-through seawater maintained at 28°C ± 0.5°C and photoperiod of 14 h light to 10 h dark. Broodstock were fed to satiation on a maturation diet of commercial pellet, squid (*Nototodarus sp.*), ox liver and green-lipped mussels (*Perna sp.*) (Wood et al. 2011). Females were unilaterally eyestalk ablated and then from two days post-ablation, females with ripe ovaries were placed in individual spawning tanks with 28°C ± 0.5°C flow-through water, oyster mesh false tank base and five glass trays to collect eggs. An automated spawning detection system (Coman et al. 2003) was used to detect spawning initiation and marked the zero time post-spawning detection (psd). Up to four pressure treatments were performed for each of the 34 families collected, by using separate 1 L small pressure chambers (SPC) (Aquatic Eco-Systems Inc., FL, USA), following a similar method to that used to induce triploidy in *Marsupenaeus japonicus* in this study (Chapter 3). At 7 min psd, the zygotes (including unfertilised eggs) suspended in 1L of seawater, were gently poured into each pressure chamber including a control chamber of same dimensions but not pressurised. At 8 min psd, an 18 tonne hydraulic press was used to manually pressurise each treatment chamber to the desired pressure between 6.9 MPa (6895 kPa) and 20.7 MPa (20,684 kPa) in 5 sec. The pressure level was maintained for 10 min and then depressurised in <1 sec by releasing the hydraulic jack. All treatment and control zygotes (including unfertilised eggs) in seawater were gently poured into separate 10 L containers and maintained at 28°C ± 0.5°C with gentle aeration for 24 h.

Hatch rates were calculated 24 h psd by counting a random sample of 300 progeny (including unfertilised eggs) under a stereo-dissecting microscope (Foote et al. 2012). Seventy-five to 100 nauplii per control and treatment were sampled separately and frozen in liquid nitrogen for later ploidy analysis using a Cell Lab Quanta SC MPL (Brea, CA, USA) flow cytometer. Each sample from the seven fertile families was processed with the flow cytometer, following a similar method to that described previously (Wood et al. 2011; Foote et al. 2012), which equalled or exceeded the standards outlined by Sellars et al (2010): shrimp cells stained with a propidium iodine solution (MPBS:11.0 g L^{-1}NaCl,0.2 g L^{-1}KCl,1.15 g $L^{-1}$$Na_2HPO_4$ containing 0.1% triton X-100, 0.2mgmL^{-1}RnaseA,0.02mgmL^{-1}PI), aspirated with a 25 G syringe and filtered though a 63 µm mesh screen and included an internal control of glutaraldehyde fixed chicken red blood cells. The data output was then analysed using the ‘modfit’ function in the FCS express 4 (De-Novo-Software 2012) software package.

The proportion of triploid nauplii produced from the total number of available fertilised and viable eggs (determined from the control hatch rate) was defined as the ‘triploid nauplii production efficiency’ and calculated as:

$$\left(Treatment\ triploid\ nauplii\ percent \times \left[\frac{treatment\ hatch\ rate}{control\ hatch\ rate}\right]\right) \times 0.01$$

5.2.2 Penaeus monodon pressure tolerance

The pressure tolerance of 60 *P. monodon* female broodstock (90g ± 10 g) was determined to evaluate if females could be left inside the large pressure chamber (LPC) post-spawn, without being killed. Shrimp were subjected to 0, 13.8, 17.2, 19, 20.7 or 27.6 MPa for a 10 min duration, using the same pressure chambers and methods as described above. A single shrimp was placed in one of the four 1 L pressure chambers in 28°C ± 0.5°C seawater. Control treatments were performed in the same manner as above, with individuals placed into the chambers for the same duration; however, no pressure was applied. Each treatment was repeated with 10 individuals. Immediately after treatment shrimp were placed in individual 10 L containers with 28°C ± 0.5°C seawater and aeration. The activity of each shrimp following treatment was observed after picking up the individual and releasing it while suspended in the water three times and classed as having either ‘normal activity’ (swimming as before the treatment), ‘reduced activity’ (laying on its side with only pleopod movement), or ‘nil activity’. This procedure was repeated 15 min post-treatment (PT), 1 h PT and 48 h PT. After 2 h PT any shrimp still not moving and showing signs of rigor mortis was determined to be dead and remaining shrimp were transferred to 80 L tanks with aerated, flow through seawater maintained at 28°C ± 0.5°C. Shrimp treated with the same pressure level were stocked together at up to three per tank and fed on a diet of squid and commercial pellet as described above for 48 h.

5.2.3 Engineering the large pressure chamber

The LPC was custom engineered to allow sufficient space for *M. japonicus* and *P. monodon* to spawn inside and then pressurise the zygotes to optimised triploid induction levels. The cylindrical LPC had an internal volume of 110 L, with a diameter of 550 mm and a depth of 610 mm. The LPC was constructed of machined high carbon steel, rated to 20.7 MPa and hard chromed for corrosion resistance. The cylindrical body of the LPC (Fig. 1i) was sealed with two large flat end ports fastened with a series of high tensile steel bolts (Fig. 1ii & iii). A smaller 120 mm port at the top of the chamber provided quick access to the interior (Fig. 1iv). In order to further combat corrosion, the bottom port was fitted with an acetal liner and stainless port fittings. All ports are fitted with high durometer O-rings to provide a water and air tight seal. The entire chamber was fitted within a steel frame 1.65m long by 0.85m wide for support, and to provide adequate access to the necessary chamber ports (Fig. 1v).

The LPC was fitted with two ports in the bottom of the chamber to facilitate water circulation (Fig. 1vi), a third port at the top for purging excess air during pressurisation (Fig. 1vii), and a final port on the side to allow for harvesting of nauplii larvae (Fig. 1iix). All ports with the exception of the larval exit port were configured with Hoke high pressure 34.5 MPa rated ball valves to allow for complete pressure isolation of the chamber. On the bottom, one port is fitted to the water supply, providing a means of filling the chamber, while the other is connected to the drain. The drain port is also fitted with a low pressure pneumatic source, which allows for aeration of the chamber if desired. The larval exit port on the side of the chamber was specially designed with smooth and seamless fittings to minimise flow turbulence, and hence larvae damage during their removal from the chamber. As such, a conventional valve could not be used. During pressurisation a plank threaded nut fitted with O-rings to maintain chamber pressure. This nut is then replaced with a smooth tube transition fitting which allows for harvesting of the nauplii larvae. Finally, a pair of threaded inserts in the bottom of the chamber allow for the installation of in-situ instruments within the chamber during pressurisation.

The LPC was pressurised using an electric air compressor driving a pneumatically driven water pump. A 2.2 kW Haskel pneumatic pump was used, and driven at 0.6 MPa and 1.7kL min^{-1} flow capacity. One half inch stainless lines were used to connect the pump to the chamber. To reduce the effect of noise, the air compressor and pump were mounted in a separate room to the chamber. Automated pressure

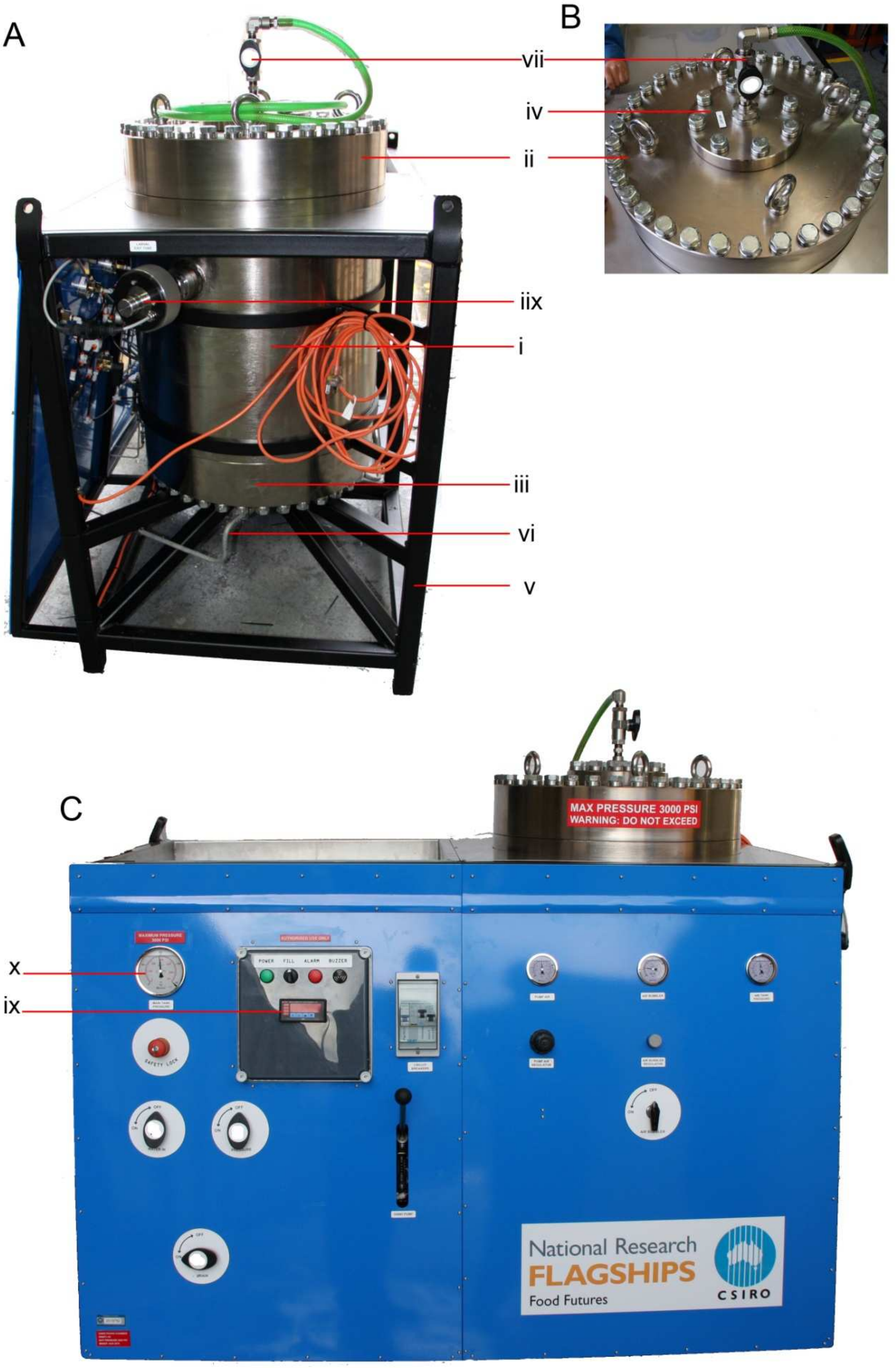

Fig. 1. Large hydrostatic pressure chamber (LPC) that was custom engineered to allow shrimp to spawn in the chamber and treat entire spawnings of eggs: side view (A), top view (B) and front view (C). The 110 L pressure chamber cylinder (i) was engineered with flat end ports with high tensile bolts (ii & iii), 120 mm quick access port (iv), steel frame (v), water flow port (vi), air purge port (vii) and larval exit port (iix). Automated pressurisation controls (ix) along with a digital (ix) and analogue (x) pressure gauge ensured the desired pressure was achieved.

controls allowed for pressurisation to a pre-programmed level (Fig. 1ix), with digital and analogue pressure gauges for the monitoring of system pressure (Fig. 1ix, x).

Once a target pressure is set the system can be programmed to maintain system pressure within 1% of the pressure setting. As a failsafe, the system is also programmed to stop pressurisation if the pressure approaches the maximum system pressure. A mechanical safety valve is also equipped in the event the electronic failsafe malfunctions.

5.2.4. Large pressure chamber inductions

Ninth generation domesticated *P. monodon* broodstock (~90 g) were used for initial trials with the LPC system by following a similar protocol to that outlined below. The initial trials with *P. monodon* utilised the LPC equipped with a 0.37 kW pump and 2.4 kW air compressor. However due to the poor results and slow pressurisation (3 min to 4 min to reach 20.7 MPa) the LPC was modified for subsequent trials. A larger diesel air compressor (3.7 kL min^{-1} flow capacity) was used to drive a 2.2 kW pneumatic pump, reducing the time to pressurise the LPC to 17.2 MPa to just 21± 1 s.

Following modification of the LPC, availability of *P. monodon* broodstock was low; therefore subsequent LPC trials were performed using wild *M. japonicus* broodstock which were seasonally available. The *M. japonicus* broodstock (~40 g) were fed the same diet and maintained in the same conditions as previously described (Foote et al in review). Impregnated females with ripe ovaries (Crocos and Kerr 1983) were unilaterally eye-stalk ablated and at least two days after ablation, ripe females were placed into the LPC to spawn.

The LPC was set up to receive a constant supply of 0.5 L min^{-1} of 28°C ± 0.5°C seawater, flowing in through the water inlet port in the base of the LPC and out through the larval exit port on the side of the chamber (Fig. 1A); allowing a constant water level to be maintained. The top access cap was removed and the spawning detection system (Coman et al. 2003) placed inside the LPC, as described above, this system was used to determine the zero time psd. The accuracy or delay in detection of spawning initiation by this device was calculated by using a stereo-dissecting microscope to observe the time psd when >50% of zygotes (from a sample of 50-100) complete first and second cleavage and comparing this to known 2 and 4-cell division times for *M. japonicus* (Hudinaga 1941).

On detection of a spawn, the larval exit port was closed and the water-in valve was opened wider to allow seawater to fill to the top of the chamber by 6:00 min psd. At 5:30 min psd a control aliquot of 5 L of eggs and seawater were gently removed from the chamber and maintained at 28°C ± 0.5°C. At 6:00 min psd the chamber lid was secured by using an air impact wrench to tighten 8 high tensile bolts. By 6:30 min psd the chamber was full and seawater was exiting the air purge port, the air purge port was and water inlet ports were then closed and the time taken to fill the chamber was recorded. With the system filled with water at line pressure, the pneumatic system was switched on at 7:00 min psd, initiating 28°C ± 0.5°C seawater to be pumped into the chamber until the target pressure of 17.2 MPa ±1% is reached by 7:21 min ± 1 sec psd, at which stage the pump stops. A treatment pressure of 17.2 MPa was applied to each of the six families treated, as this pressure level was previously found to be optimal for *M. japonicus* (Chapter 3). The pump valve was then closed to completely isolate the chamber and maintain the pressure. Once the desired pressurisation time is of 10 min was reached, the pressure was relieved by opening the air purge valve at 17:00-17:10 min psd. The access cap was then removed and the female spawner removed from the LPC and placed in an 80 L holding tank, where the activity level of the spawner was observed.

An ‘external treatment aliquot’; 10 L of embryos and seawater, was taken from the chamber at 2 h psd in families 4-6 using the same method as the control aliquot. Flow through seawater was supplied to the LPC by opening the water-in valve to allow a flow of 0.5 L min^{-1}. The larval exit port was then opened and a 60 µm mesh screen inserted to prevent swimming nauplii from exiting prior to sampling. Gentle aeration was added to the chamber as well as the external treatment and control aliquots 1 h psd and all were maintained at 28°C ± 0.5°C. Hatch rates and ploidy analysis was performed in the same manner as described above.

5.3. Results

5.3.1 Penaeus monodon triploid optimisation using small pressure chambers

Of the 34 *Penaeus monodon* families, 20 were infertile, and seven were detected more than five minutes late as determined by observing the timing of the cell divisions. Of the remaining seven families, triploid stage 1 nauplii were detected in 24 of 25 treatments (Table 1). Analysis of variance (one-way ANOVA) revealed that pressure had a significant effect on triploid induction (P=<0.00001). Triploid rates typically increased as pressure increased (Fig. 1A) with a rate of 100% triploid nauplii achieved from three of six treatments at 17.2 MPa and two of three treatments at 20.7 MPa (Table 1). The greatest rates of triploid nauplii induction were also achieved following pressure levels at 17.2 MPa and 20.7 MPa, with mean triploid rate of 84.6% and 97.2% at these levels respectively; triploid rates following these two pressure levels were significantly different to all other pressure levels (LSMEANS). There was no significant family effect on triploid induction levels (one-way ANOVA: P=0.3820).

Pressure level also had a significant effect on hatch rates relative to controls (one-way ANOVA: P=<0.0001), which generally decreased as pressure increased. The highest mean relative hatch rate was 83.7% following treatment at 6.9 MPa (Fig. 1B), with hatch rates at this level significantly different to all other pressure levels. Mean relative hatch rates were below 25% following pressure levels 13.8, 17.2 and 20.7 MPa; hatch rates following these pressure levels were significantly different to the other pressure levels (LSMEANS) and as low as 1.4% on one occasion at the highest pressure of 20.7 MPa (Table 1). There was also a significant family effect on hatch rates relative to controls (P=0.0372)

The most efficient triploid nauplii production rate was at 17.2 MPa with a mean value of 15.2% (Fig. 1C). The least efficient rate of triploid production was achieved following treatment at 20.7 MPa, with a mean of 7.7% of viable progeny hatching into triploid nauplii at this level (Fig. 1C.). However, the effect of pressure on triploid production efficiency was not significant (one-way ANOVA: P=0.3192), there was also no significant family effect on triploid production efficiency (P=0.8641).

Table 1: *Penaeus monodon* triploid induction, hatch rate relative to control and production efficiency following treatment with hydrostatic pressure for seven families

Pressure (MPa)	Family	3N nauplii (%)	Hatch rate relative to control[a]	Production efficiency[b]
6.9	4	14.2	63.07	15.8
	5	5.2	90.3	13
	6	7.3	97.63	9.6
10.3	*1*	*12.6*	5.72	0.9
	2	0	35.29	0
	3	22.2	80.66	29.6
	4	13.3	58.83	13.8
	5	8	74.72	16.5
	6	30.7	54.14	2.5
13.8	1	86.6	6.48	7.1
	2	30.5	8.4	10.8
	3	45.9	29.4	22.3
	4	35.3	19.08	11.9
	5	*43*	*38.47*	45.6
	6	46.1	38.24	23.9
	7	30.1	31.25	15.4
17.2	1	100	17.92	22.8
	2	100	27.31	114.8
	3	59.5	25.34	24.9
	4	100	5.65	10
	5	52.8	13.67	19.9
	6	95.3	31.8	41.1
20.7	1	91.6	1.4	1.6
	3	100	28.69	47.4
	7	100	2.5	4.1

[a]hatch rate relative to control calculated as:
$(treatment\ hatch\ rate/control\ hatch\ rate) \times 100$ $(\mathrm{n} = 300\ \mathrm{progeny})$
[b]production efficiency calculated as:
$(Treatment\ triploid\ nauplii\ percent \times [treatment\ hatch\ rate/control\ hatch\ rate]\ ) \times 0.01$

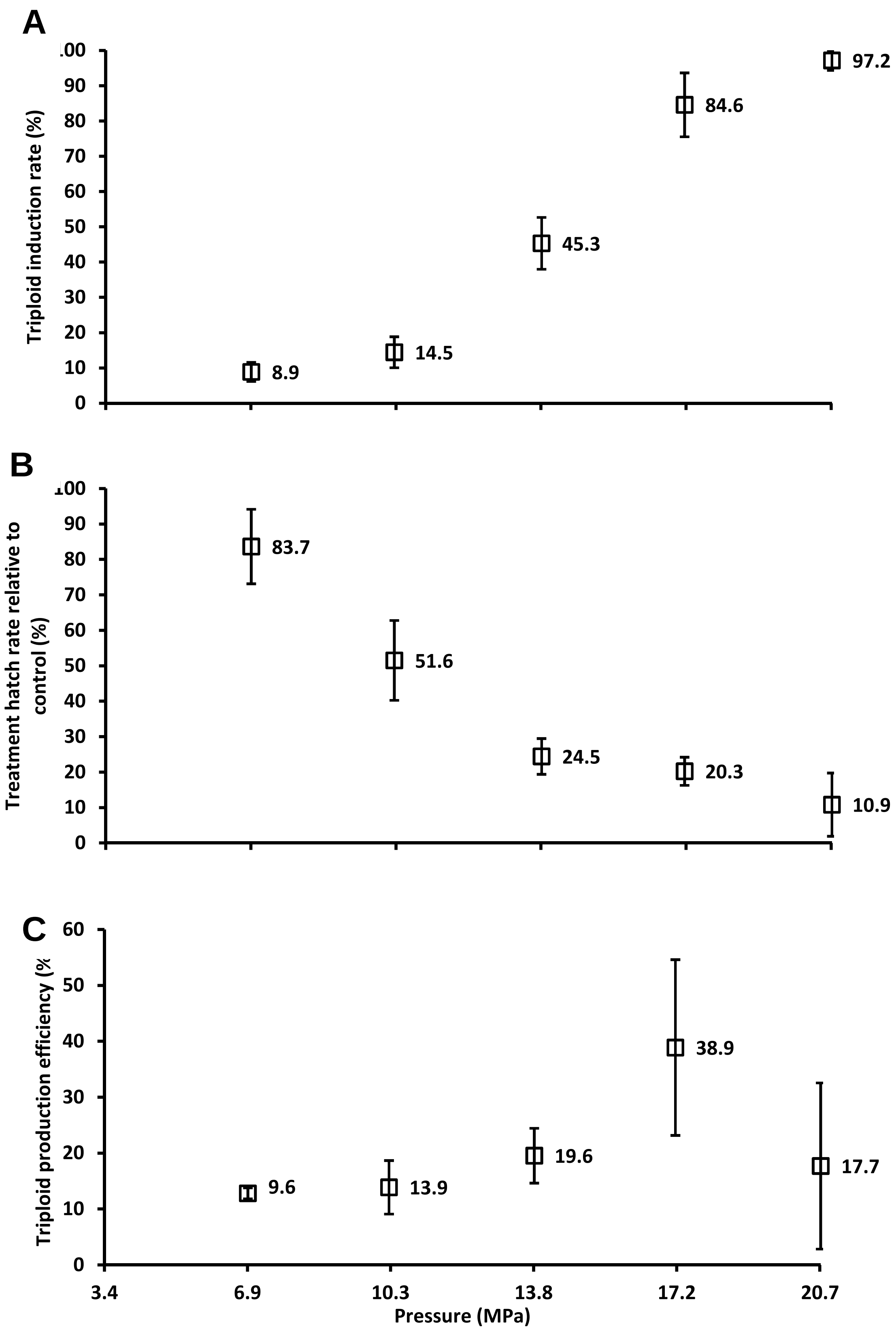

Fig. 2. *Penaeus monodon* triploid nauplii mean induction rate (A); mean hatch rate relative to control $(treatment\ hatch\ rate/control\ hatch\ rate) \times 100\ (\mathrm{n} = 300\ \mathrm{progeny})$ (B); and triploid production efficiency $(Treatment\ triploid\ nauplii\ percent \times [treatment\ hatch\ rate/control\ hatch\ rate]\ ) \times 0.01$ (C). A-C verses pressure treatment following 25 treatments from seven families, with standard error bars.

5.3.2 Penaeus monodon pressure tolerance

None of the *P. monodon* broodstock treated with 0 MPa, 13.8 MPa and 17.2 MPa showed any obvious decrease in activity following treatment and were therefore classed as having ‘normal activity’, with all shrimp swimming when released in the water column (Table 1). In contrast, all of the shrimp that were treated with 19 MPa, 20.7 MPa and 27.6 MPa showed ‘reduced activity’ 15 min following treatment, with no shrimp actively swimming following release into the water column; all shrimp sank to the bottom of the tank and were laying on their side either not moving at all or with limited pleopod movement (Table 1). Some of the shrimp treated at these high levels showed increased activity levels by 1 h and 48 h post-treatment. However, the majority of shrimp treated at 19 MPa, 20.7 MPa and 27.6 MPa did not recover and died by 48 h PT: 70% following 19 MPa and 80% following 20.7 MPa and 27.6 MPa (Table 1). In summary, pressure treatments at levels up to and including 17.2 MPa resulted in no obvious reduction in broodstock activity 48 h post-treatment but pressures above this level induced increasing levels of mortality.

5.3.3 Large pressure chamber inductions

Initial trials of the LPC with *P. monodon* resulted in inconsistent triploid induction rates and low hatch rates. Triploidy was successfully induced in three of eleven fertile families, with the highest induction rate at 57.7%. However, the hatch rate of these three families was very low, the highest being 2.6% (data not shown).

Subsequent trials of the modified LPC successfully produced triploid nauplii in all six *M. japonicus* families following pressure treatment, with triploid induction rates varying from 59.6% to 100% (Table 2). Both control and treatment hatch rates varied between families, with hatch rates following pressure treatment lower than their respective controls for all families (Table 2). Due to the large variability in the control hatch rate between families, the treatment hatch rate relative to the control hatch rate was calculated for each family. This allowed the drop in hatch rate due to the treatment and ploidy induction to be compared, with relative hatch rates between 5.1% and 77.5%. In families 4, 5 and 6, relative hatch rates taken from treated progeny inside the chamber as well as treated progeny transferred to external aliquots at 2 h psd were calculated; external aliquots were 42.5% (family 4), 45.4% (family

5), and 46.3% (family 6) lower than aliquots remaining in the chamber. In summary, high to absolute induction of triploids was achieved in the LPC, hatch rates in treated aliquots were lower than control aliquots and hatch rates were further reduced when aliquots were removed 2 h psd.

The delay in detection of spawning initiation was calculated based on known cell division times (as mentioned above), with induction rates highest when the delay in detection of spawning initiation was shortest; families 1, 3 and 4 were detected with the shortest delay (<1 min) and had the three highest induction rates (Table 2). Protozoea were reared in Family 6 which had the highest hatch rate, triploids were detected at this stage at a rate of 20.2% (Table 3).

The time taken to fill the chamber with seawater was 6 min for families 1, 4, 5 and 6 while in families 2 and 3 the fill time was 3 min (Table 2). In families 1 and 3, which had different fill times, triploid induction rates of 100% was achieved in both instances; however, hatch rates relative to the control were significantly different: 28.2% versus 5.1% respectively.

The effect of the pressure induction on the activity level of the spawner was also noted. None of the 12 *M. japonicus* broodstock that remained in the LPC during treatment of 17.2 MPa were moving 2 min following treatment when the lid was opened, allowing the broodstock to be easily removed. All broodstock then recommenced swimming within an hour after relocation into an 80 L holding tank.

Table 2: Activity level for 10 *Penaeus monodon* broodstock per treatment pressure level, before and after treatment with pressure shock, to determine the maximum survivable pressure level

Treatment pressure level (MPa)	Time before or after treatment									
	0:15 h before treatment	0:15 h post-treatment			1:00 h post-treatment			48:00 h post-treatment		
	Broodstock activity level									
	Normal[a]	Normal[a]	Reduced[b]	Nil[c]	Normal[a]	Reduced[b]	Nil[c]	Normal[a]	Reduced[b]	Nil[c]
0	10	10			10			10		
13.8	10	10			10			10		
17.2	10	10			10			10		
19	10		3	7	3	1	6	3		7
20.7	10			10		2	8	2		8
27.6	10			10		2	8	2		8

[a] Normal broodstock activity defined as an individual swimming following release in the water column
[b] Reduced broodstock activity defined as an individual not swimming following release in the water column, but showing some pleopod movement while laying on its side
[c] Nil activity defined as no appendage movement following release in the water column, after 2 hours these shrimp were rigor mortis and determined to be dead

Table 3: Large pressure chamber triploid induction rate, hatch rate and hatch rate relative to control for *Marsupenaeus japonicus*

Family	Percent triploid (%)		Control hatch rate (%)	Treatment hatch rate (%)		Hatch rate relative to control (%)		Delay in spawning detection (min)[c]	LPC fill time prior to pressurisation (min)
	Nauplii	Protozoa		Chamber[a]	External[b]	Chamber[a]	External[b]		
1	100	-	74.2	20.9	-	28.2	-	<1	6
2	72.7	-	36.1	7.7	-	21.3	-	1	3
3	100	-	53.1	2.7	-	5.1	-	<1	3
4	93.5	-	78.2	16.2	8.7	20.7	11.1	<1	6
5	59.6	-	89.1	16.3	8.9	18.3	10.0	3	6
6	29.5	20.2	95.7	74.2	42.7	77.5	44.6	5	6

[a] aliquot of 350±50 eggs/nauplii sampled from inside the chamber 24 h psd
[b] aliquot of 350±50 eggs/nauplii sampled 24 h psd from an aliquot external to the chamber; 8 L of eggs and seawater siphoned from the LPC 2 h psd and maintained at the same temperature and aeration as the LPC
[c] determined by using a dissecting microscope to observe the time (psd) >50% of zygotes (from a sample of 50-100) complete first and second cleavage and comparing this to known 2/4-cell division times

5.4. Discussion

5.4.1. Pressure technique produces triploid Penaeus monodon (SPC)

Triploid *Penaeus monodon* nauplii were successfully produced by using small pressure chambers (SPC) to generate hydrostatic pressure and inhibit meiosis II. This technique was recently used to induce triploidy in *Marsupenaeus japonicus* (Chapter 3); however, the transfer of the technique to *P. monodon* in the current study, to create reproductively sterile stocks is important, due to the significant genetic improvements achieved in this species and the commercial significance of *P. monodon*, particularly in Australia (Glencross et al. 2010; Preston et al. 2010; Glencross et al. 2012).

Triploid *P. monodon* nauplii were produced at a similar pressure range to that previously determined in *M. japonicus* (Chapter 3), with triploids produced in both species following pressure shocks at levels of 13.8 MPa, 17.2 MPa and 20.7 MPa. However, lower hydrostatic pressure levels of 6.9 MPa and 13.4 MPa only generated triploid nauplii in *P. monodon*. The highest levels of triploidy were achieved in *P. monodon* following treatment at 17.2 MPa and 20.7 MPa, with induction rates of 100% triploidy at these two pressure levels (following five of nine treatments). Previous optimisation of pressure shock treatments for induction of triploidy in *M. japonicus* also found that 100% triploid induction was achieved at 17.2 MPa (Chapter 3). The production of triploids at absolute rates using hydrostatic pressure in *P. monodon* and previously in *M. japonicus*, demonstrates the potential of this technique to create stocks of reproductively sterile shrimp.

Hatch rates in *Penaeus monodon* decreased as pressure and triploid induction rates increased; however, it is unclear what proportion of mortality is due to inhibition of meiosis and the implications of becoming triploid or due to the pressure shock adversely affecting the zygote in another way. Hatch rates following triploid inductions using SPCs in *M. japonicus* were also lower than controls in treatments at 13.4 MPa and above; however, hatch rates were generally higher in *M. japonicus* at 17.2 MPa than at 13.4 MPa and 13.8 MPa; this spike in hatch rates at 17.2 MPa could not be explained (Chapter 4) and was not reflected in the current study with *P. monodon*. Handling stress resulting from transferring zygotes to and from the SPC also increased mortality rates, a finding common with other ploidy manipulation studies (Sellars et al. 2012b). In addition, the most efficient production of triploid nauplii was achieved at 17.2 MPa in both *P. monodon* and *M. japonicus* (Chapter 3); this optimal level was more distinct in *M. japonicus*.

5.4.2. Triploid production at absolute rates (LPC)

Triploid *M. japonicus* were produced in this study at high or absolute rates in the LPC, provided the optimal treatment pressure of 17.2 MPa was applied at the precise time required to inhibit meiosis II. Successful triploid production from LPC inductions repeat the successful induction rates achieved with SPC inductions in *M. japonicus* (Chapter 3), where high and absolute induction rates were also achieved following treatment at 17.2 MPa. The time required to pressurise the LPC was longer than in SPC; taking 21±1 s to pressurise the LPC to 17.2 MPa, rather than 5 s in the SPC. However, this longer time to pressurise the LPC doesn't appear to have limited induction success. The production of triploids at absolute rates using the LPC has demonstrated that entire families of reproductively sterile triploid shrimp can be produced, potentially guaranteeing genetic protection of these families.

5.4.3. Treating whole spawnings (LPC)

While hydrostatic pressure was used to successfully produce triploids SPCs were not suitable for commercial application as only part of a spawning was treated and the technique involved handling of fragile zygotes which increased mortality. While some attempts have been made previously to produce triploids on a large scale by treating whole spawnings (Sellars et al. 2012b), the techniques still resulted in handling stress when transferring zygotes in and out of treatments. These techniques could not reliably produce triploids at high or absolute rates and the treatment techniques are labour intensive, which is not commercially desirable. The hydrostatic pressure technique has allowed a more practical engineering solution to be developed to treat an entire shrimp spawning; a LPC was engineered to allow shrimp to spawn inside the chamber then treat the entire spawn. The induction parameters determined using SPC inductions were successfully transferred to the LPC to produce triploids.

Further modifications to this prototype system could also increase automation to permit large scale triploid induction for commercial adoption.

5.4.4. Eliminating handling stress (LPC)

Triploid inductions using the LPC successfully eliminated the need to handle progeny at their earliest, most fragile stages of development; with progeny remaining in the LPC from the time they are released from the female, until hatching into swimming nauplii. The effects of handling stress on *M. japonicus* later in embryogenesis (2 h psd) was demonstrated in this study, as hatch rates decreased when an aliquots of embryos were removed from the LPC at 2 h psd, with hatch rates more than 40% lower than in the corresponding aliquot that remained inside the LPC. These findings emphasise the importance of eliminating handling stress to reduce mortality even at later stages of embryogenesis. While retaining the treated embryos in the chamber until hatched into nauplii (hatching occurring 12-14 h psd) will maximise hatch rate, the trade off is that only one spawning per day can be treated in the LPC.

5.4.5. Pressure treating spawners

The pressure tolerance of female *P. monodon* broodstock was determined to evaluate if they could be left inside the large pressure chamber (LPC) during treatment without being killed: an important requirement since the technology is being developed to be used on highly valuable broodstock. Knowledge of pressure tolerance would also assist in engineering a prototype LPC. For example, if it is found the female needs to be removed to avoid death from pressure, the lid would need to be large enough to allow sufficient access to remove the spawner; however, the increased weight of a larger lid would require further engineering solutions to lift and secure this lid in the short amount of time required.

The tolerance of *P. monodon* broodstock to pressure stress revealed that treatment at pressure levels of 17.2 MPa did not kill the shrimp indicating that it may be acceptable to leave spawners inside a LPC. The affects of pressure stress on *M. japonicus* spawners was also tested when treatments at 17.2 MPa were performed in the LPC, during triploid induction of their progeny. All females survived for at least 48 h following treatment despite a short period of reduced activity; however, the long term effects of this pressure treatment on survival and reproductive output are yet to be quantified.

It was decided that the spawners would remain inside the chamber during the pressure treatment as it was more practical and would reduce stress on the eggs/zygotes; the short window of time available to remove the broodstock combined with the limited amount of chamber access meant it would be difficult to successfully remove the spawners prior to pressurisation and attempts to catch and remove the spawners would likely cause damage to the eggs/zygotes. From a commercial perspective, leaving the shrimp in the pressure chamber would be more desirable as it would require less labour and with a more automated system, shrimp removal would not be practical.

5.4.6. Persisting issues and potential solutions

Further research should focus on addressing issues which are common to all treatment methods in previous ploidy manipulation studies in shrimp and continue to prevent reliable triploid induction at high or absolute rates. The first issue is the accuracy of spawning detection, as late treatment application appears to be the major issue preventing high rates of triploid nauplii production in several families, in the LPC trials. Therefore, by developing methods of detecting a spawn with greater accuracy, high or absolute induction rates could be achieved more reliably. One potential detection method may involve the use of an optical sensor which could be readily integrated into the LPC though one of the dedicated ports in the base of the chamber and could withstand pressurisation. An additional advantage in using an integrated device would be the ability to keep the chamber sealed, eliminating the need to secure the cap prior to pressurisation, also improving the potential for automating the system.

One of the hardest issues to address, which continues to limit the reliability of ploidy induction in penaeid shrimp, is the fundamental characteristic of unsynchronised development of penaeid shrimp progeny and thus the inability to apply treatments to the entire spawn at precisely the same developmental stage. This is a result of the female releasing her eggs over a period of up to several minutes, with egg activation occurring upon contact with seawater (Clark and Lynn 1977; Pongtippatee-Taweepreda et al. 2004). The consequence of which is that pressure shocks are applied to progeny at a range of developmental stages within a single spawning event, resulting in shocks being applied at sub-optimal times, potentially missing meiosis II. One study claims to address this issue by spawning shrimp in magnesium deficient seawater, which was reported to suspend embryonic development until normal levels of magnesium are restored, resulting in synchronised development (Pongtippatee et al. 2010). Unfortunately, these results could not be repeated (CSIRO, unpublished) and no further studies published after this have reported using this method to improve synchronisation of development. If developmental synchronisation can be reliably achieved it has the potential to improve the reliability of successful triploid inductions and could also allow triploidy to be achieved following shorter treatment durations thereby potentially improving hatch rates. Furthermore, as egg activation occurs upon contact with seawater, not fertilisation by sperm penetration (Clark and Lynn 1977; Pongtippatee-Taweepreda et al. 2004), artificial fertilisation cannot provide a solution to synchronising development.

Despite the LPC eliminating the need to handle progeny, hatch rates were still lower than control hatch rates even though control eggs had to be handled (removed from the LPC) to avoid treatment. Results of SPC inductions indicate that there is some mortality due to the effects of pressure stress early in development and potentially meiosis II inhibition and becoming triploid. Anecdotal evidence suggests that a large proportion of the reduced hatch rate from treatments might be attributable to another physical stress, resulting from the way the prototype LPC system was designed. The LPC needed to be filled with approximately 12 L of seawater in a short amount to time prior to pressurisation and by filling the LPC with seawater though a small inlet in the base of the LPC, a large amount of water turbulence resulted. By comparing the two families which both had 100% triploid induction rates but significantly different hatch rates relative to the control (28.2% versus 5.1%). As all the parameters were the same except for fill rate, it could be inferred that stress from the water turbulence might have been reducing the hatch rate as the family with the lower hatch rate was filled with water twice as fast as the family with the higher hatch rate. If a future LPC design incorporates an integrated spawning detection system as mentioned above, then the LPC could remain completely filled during a spawning event, with water exiting out the air purge valve, this would eliminate the need to fill the chamber prior to pressurisation. Further trials with the LPC should also focus on optimising the treatment duration, as previous studies have found that reducing treatment duration may reduce mortality (Chapter 3). However, reducing treatment duration has to be balanced carefully with maintaining high triploid induction rates.

5.4.5. Conclusion

This study has made significant progress in developing a prototype system that could allow triploid shrimp production in a commercial hatchery; this could ultimately be used to prevent unlicensed breeding of genetically improved lines of shrimp. The prototype LPC successfully resolved some of the major issues currently limiting triploid production commercially; allowing treatment of all the progeny from *M. japonicus* spawnings, producing triploids at high or absolute rates and eliminating the need to handle the progeny to apply or remove the treatment. This study also demonstrated that the hydrostatic pressure technique could be transferred to *P. monodon*, a commonly farmed shrimp, with genetically improved lines achieved in several hatcheries. Optimal induction pressure was determined with *P. monodon* using small pressure chambers and triploidy achieved at high and absolute rates; triploid inductions of *P. monodon* can now trialled in a LPC.

Following integration of an improved spawning detection system, this technique would then be suitable for trials in a commercial hatchery with the aim of producing large quantities of triploids. If the issue of unsynchronised egg activation can be addressed, the reliability of triploid inductions at high or absolute rates, with higher hatch rates could be improved.

Authors' Contributions

Andrew Foote was responsible for experimental design, implementation of experiments and writing this chapter, as well as assisting with prototype large pressure chamber design and broodstock husbandry. Andrew Wood assisted with pressure chamber operation, broodstock husbandry and revision of an early draft.

Andreas Marouchos, Dave Kube and the CSIRO Marine and Atmospheric Research, Science Engineering and Technology workshop engineered and installed the prototype large pressure chamber. Melony Sellars and Graham Mair provided conceptual, logistical and editorial support.

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CHAPTER 6

General Discussion

In the absence robust legal or business solutions to protecting the IP invested in genetically improved shrimp lines, the shrimp industry demands that methods of genetically protecting improved shrimp lines be developed before such lines are sold on to other farmers. Development of such a method would enable the benefits from improved shrimp lines to spread more widely, speeding up the impact of genetic programs on the industry globally. Triploidy currently provides the only avenue to guarantee reproductive sterility and in addition, triploidy could also improve production in some species where triploidy skews sex ratios to the faster growing female sex (Hansford and Hewitt 1994; Coman et al. 2004).

While triploid shrimp families have been reared to adults (Coman et al. 2008; Sellars et al. 2012a), triploid induction techniques trialled prior to this study are not suitable for commercial adoption. The research presented in this thesis sought to address current problems limiting commercial production of triploid shrimp, by progressively trialling innovative induction techniques that could induce triploidy in whole spawnings, allowing large quantities of triploids to be produced, while also improving induction rates and reducing mortality from handling stress.

6.1. Tetraploid induction

Production of tetraploid shrimp broodstock is one avenue that could potentially resolve the current issues associated with direct induction of triploids though polar body inhibition, preventing commercial production of triploidy. Current limitations include low induction rates and production of small quantities of triploids. Whole families of triploids may be achieved by mating tetraploid and diploid broodstock, as reported in oysters (Guo et al. 1996; Wang et al. 2002) and Rainbow Trout (Chourrout et al. 1986). Furthermore, triploids produced from tetraploid and diploid crosses may also have improved hatch rates compared to direct induction, as there would be no need to handle progeny to directly induce triploids. Finally, triploid production though spawnings of tetraploid and diploid crosses would be more practical for commercial adoption compared to current triploid induction techniques, as direct induction of triploidy though meiotic inhibition is currently required for each spawning/family.

However, to date there have been no reports of viable tetraploid shrimp production, with heat shock the only treatment agent trailed in shrimp to inhibit first cleavage and induce tetraploidy prior to commencement of this research project (Li et al. 2003a; Sellars et al. 2006a). Cytological analysis of heat induced tetraploid *Marsupenaeus japonicus* embryos (Foote et al. 2012) and *Fenneropenaeus indicus* (Morelli and Aquacop 2003) embryos revealed that lethal abnormalities resulted immediately following treatment and were likely a consequence of treatment with heat. Consequently, this study investigated if two alternative treatment agents could induce tetraploids and produce viable offspring; cold shock and hydrostatic pressure shock.

First, the use of cold shock to induce tetraploidy was trialled as cold shock had recently proven successful at producing triploid shrimp (Wood et al. 2011). The current study found that cold temperatures (-2 °C to 1 °C) could be used to induce tetraploidy in *Penaeus monodon*; however, the treatment agent was not suitable as the cold temperatures required to induce tetraploidy were lethal to all treated progeny, both diploids and tetraploids.

Second, hydrostatic pressure was used as a novel treatment agent to induce tetraploidy in shrimp. Hydrostatic pressure was chosen due to its success in manipulating ploidy in a large range of marine fish (Onozato 1984; Chourrout 1986; Hussain et al. 1993; Goudie et al. 1995; Johnstone and Stet 1995; Malison and GarciaAbiado 1996; Peruzzi and Chatain 2000) and marine invertebrates (Chaiton and Allen 1985; Arai et al. 1986; Komaru and Wada 1989b; Shen et al. 1993; Ding et al. 2007), as well as its reported ability to overcome lethal aberrations resulting from heat shock in Rainbow Trout, *Oncorhynchus mykiss* (Zhang et al. 2007). Consequently, this study sought to determine if hydrostatic pressure could also prevent aberrations early in development, and determine if viable tetraploids could be produced.

Tetraploidy was induced in *M. japonicus* at rates of up to 100% following treatment with hydrostatic pressure, using a range of induction parameters. However, no tetraploid nauplii were detected on any occasion and cytological analysis of DNA stained embryos revealed the same lethal trait previously observed with heat induced tetraploid *M. japonicus* (Foote et al. 2010); anucleate cells from the 4-cell stage in tetraploid embryos, which was not resolved later in development.

If tetraploid shrimp abnormalities that resulted as a side effect of first cleavage inhibition in the current and previous studies (Morelli and Aquacop 2003; Foote et al. 2010) can be resolved, the elevation in ploidy status to tetraploidy in penaeid shrimp might still be lethal, as tetraploid mortality has been commonly observed at early developmental stages in other animals. One explanation for tetraploid mortality around or just after the time of hatching may be from deleterious effects on cellular metabolism resulting from reduced cell surface area (Pandian and Koteeswaran 1998; Sakao et al. 2006). An example of non-viable tetraploids has been reported in Masu Salmon, *Oncorhynchus masou,* which die at early stages of development as a consequence of side effects from the elevation in ploidy to tetraploidy, rather than from treatment at first cleavage (Sakao et al. 2006). Sakao et al (2006) determined that tetraploid Masu Salmon, *Oncorhynchus masou*, are destined to die from side effects of elevation in ploidy, with tetraploid embryos exhibiting an inadequate vascular system and also blood congestion on the surface of the yolk sac.

It is also possible that tetraploid shrimp may be non-viable due to their high chromosome number; 88 in *P. monodon*, *Litopenaeus vannamei*, *Fenneropenaeus chinensis* and *Fenneropenaeus merguiensis* and 86 in *M. japonicus* (Sellars et al. 2010). However, viable tetraploids have been achieved in salmonids (Chourrout 1986), where the diploid number of chromosomes is 60 to 80 (Phillips and Rab 2001). Tetraploid viability in certain salmonids may be a consequence of their unique evolution from a tetraploid ancestor (Phillips and Rab 2001; Zhang et al. 2007).

The success of triploidy is usually higher than for tetraploidy and in some cases this may be explained by fundamental differences in meiotic inhibition and mitotic inhibition. Abnormal spindle architecture has been reported to result in abnormal cleavage and/or anucleate cells following inhibition of first cleavage in *M. japonicus* following hydrostatic pressure (Chapter 3) and following heat shock in *M. japonicus* (Foote et al. 2010), *F. indicus* (Morelli and Aquacop 2003) and *O. mkyiss* (Zhang et al. 2007). It has been suggested that this polypolar spindle formation is a result of splitting of mother and daughter centrioles (Zhang et al. 2007). As meiotic spindles do not contain centrioles, the formation of polypolar spindles and the resulting abnormalities would not occur following triploid inductions (Zhang et al. 2007). Instead, successful triploid induction can be achieved as normal bipolar meiotic spindle formation results following successful polar body inhibition, as observed in *Sicyonia ingentis* following treatment with cytochalasin D (Hertzler 2002).

6.2. Direct induction of triploidy

Following the failure of viable tetraploid production, innovative methods of directly inducing triploidy were investigated. Hydrostatic pressure was selected for direct induction of triploidy as the method has been used to successfully manipulate ploidy and induce tetraploidy in the current study. In addition, hydrostatic pressure has also proven its success at manipulating ploidy in a number of fishes and invertebrates (as outlined above); however, prior to this thesis there have been no reports of hydrostatic pressure trials in any crustacean. Further, hydrostatic pressure could potentially resolve some of the major issues currently preventing commercial adoption of triploidy, through the construction of a large pressure chamber (LPC). A LPC that is large enough for broodstock to spawn inside would allow treatment of an entire spawning, eliminating the handling stress associated with transferring progeny at fragile developmental stages to apply or remove the treatment.

Small experimental scale trials of hydrostatic pressure shocks were performed to determine if triploids could be successfully produced and to optimise induction parameters for large scale pressure trials. Triploid *M. japonicus* nauplii were produced following an optimal pressure treatment level of 17.2 MPa, as this level resulted in the highest mean triploid nauplii rates, highest triploid nauplii production efficiency and was the only pressure level which resulted in production of triploid nauplii at a rate of 100%. Therefore, the success of the hydrostatic pressure method in the current study prompted application of the treatment to Australia's predominately farmed shrimp species *P. monodon* and to also design and trial pressure treatments in a large scale hydrostatic pressure chamber to determine if entire families/spawnings of shrimp could be induced at an absolute rate and also eliminate handling stress which decreases hatch rates.

Optimised hydrostatic pressure shock parameters used to produce triploid *M. japonicus* were transferred to *P. monodon* to determine if triploidy could also be achieved in this species. Triploid nauplii production was successful in *P. monodon* following similar treatment parameters in the small pressure chambers (SPCs). The highest induction rates and several families of 100% triploids were achieved following pressure treatment at 17.2 MPa and 20.7 MPa in *P. monodon*. Following the success of hydrostatic pressure as a treatment technique to induce triploidy at high and absolute rates in *M. japonicus* and *P. monodon*, the technique was scaled up with the goal of treating entire families and improving hatch rates by reducing handling stress.

The optimal induction parameters from the SPC inductions of *P. japonicus* were transferred to LPC inductions, where whole families of *M. japonicus* could be treated. Consistent with the results from the SPC, triploid nauplii were produced at high and absolute rates following optimal treatment conditions. The LPC induction method is a significant breakthrough in ploidy manipulation in shrimp as the technique has allowed production of whole families of triploids and eliminated the need to handle progeny until hatched, reducing mortality due to handling stress. Following modification to the LPC with the addition of an integrated spawning detection system, high or absolute triploid induction rates may be achievable more reliably and embryo mortality may also be reduced due to the requirement of less water to fill the chamber. Induction reliability could be further improved if techniques to synchronise egg development are achieved, allowing all progeny to be treated at precisely the same developmental stage.

6.3. Conclusion

This research project progressively trialled a number of novel methods of tetraploid and triploid induction, addressing some of the major issues limiting commercial adoption of the technology, with the ultimate goal of genetically protecting improved lines of shrimp and increasing the proportion of the faster growing female sex in some species.

Successful production of tetraploidy would have provided the most commercially desirable avenue to produce triploid shrimp, whereby entire families of triploids may be produced by crossing tetraploid and triploid broodstock. However, successful tetraploid production continues to remain out of reach in shrimp, despite trialling novel induction techniques in this study.

Direct induction of triploidy in this study using the LPC provides the most successful method of triploid production to date and the greatest progress towards commercial adoption of triploidy. Following minor modifications to the LPC, it is expected that further improvements in the reliability of high or absolute triploid induction rates may be achieved along with improvements in embryo survival. The success of the LPC technique to induce triploidy may then be assessed in other shrimp species and ultimately the results of a trial in a commercial hatchery would determine if the technology will be adopted by the shrimp farming industry.

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