Commercial production of all-female reproductively sterile Giant Tiger prawns (*Penaeus monodon*): Assessing their commercial performance in ponds

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Non-Technical Summary

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OBJECTIVES:

1. Develop *P. monodon* triploid heat shock induction systems that allow all eggs spawned from any one female to be exposed to heat shock at the same time.
2. Determine the performance (growth, survival, reproductive sterility and sex ratio) of triploid *P. monodon* when reared to reproductive maturity compared to diploids.
3. Develop commercially relevant *P. monodon* triploid induction protocols for the Australian industry.
4. Transfer commercially relevant triploid induction technologies to one or more commercial hatcheries.

NON TECHNICAL SUMMARY:

Background

Several Australian companies currently have breeding programs producing domesticated and selective-bred *P. monodon* lines. The most advanced of these lines have demonstrated high commercial pond performance over several generations. Importantly, the broader distribution of these high performance lines to other grower companies poses a risk for the breeding program companies, through loss of their investment by potential on-sale or on-rearing of their selected post-larvae. One avenue to overcome broader distribution of the selected post-larvae, but without the risks to breeding companies of losing their genetic investment, is to have a fail-proof means of genetic protection. As a result, the demand for a fail-proof method of genetic protection in Black Tiger prawns has never been so great.

To-date there is no commercial method for fail-proof genetic protection of prawns. Triploid induction (which gives each individual an extra set of their own chromosomes; a process that occurs sporadically in nature) is the only methodology that has been trialled experimentally that shows promise of genetic protection in prawns. However, there are limited reports of triploid induction in Black Tiger prawns and no studies have reared the larvae through to harvest age and conducted rigorous performance measurements for this species.

Aims

This project aimed to up-scale CSIROs well optimised laboratory scale triploid induction methodologies to develop a technique that would be suitable for commercial triploid induction of whole spawnings of the Giant Tiger prawn. Close industry engagement was sought throughout the project to allow practical solutions to complex experimental and biological challenges, and to disseminate methodologies to commercial hatchery staff. The project also assessed the performance of triploid Giant Tiger prawns from egg to reproductive maturity for the first time.

Project outcomes

Up-scaling of laboratory triploid induction systems to commercially suitable whole spawning induction systems whilst maintaining high hatch rates and induction rates proved to be the key challenge of this project. Triploid induction requires a shock agent (eg. temperature or chemical) to be applied to newly spawned embryos at ~7min 30 sec post-spawning for a 10 min duration. Embryos at this age are extremely fragile and any sort of handling (i.e. for concentrating the eggs and applying the shock) can damage the embryos and compromise hatch rates.

A whole spawning induction system using a 90 µM mesh pen was developed, which allowed the successful induction of whole spawns with temperature and chemical shocks. However, hatch rates from this system were consistently low. Despite this a chemical induced triploid family with a high induction rate and reasonable larval survival was produced at a commercial hatchery, allowing rigorous tank-based performance assessments to be carried out.

The overall performance of triploids was comparable to other penaeid prawn species with some exceptions. Survival of the triploids was lower than diploids. Triploid females, triploid males and diploid males had similar weights at harvest age, with diploid female siblings being significantly larger. The triploid sex ratio was 1 female: 1.625 males. This was a unique finding as studies on other penaeid prawn species have found triploids to be predominantly female. When reared to 'reproductive age', the triploid females and males were unable to produce viable offspring when crossed with diploid siblings and wild-caught diploids. Histological examinations found that triploids had abnormal gonadal cellular structure and cellular organisation compared to diploid siblings at both harvest and reproductive age. Results from this study demonstrate that triploidy would provide the industry with a method of genetic protection for Black Tiger prawns, however significant challenges in maintaining high hatch rates, survival rates and induction rates of the triploids when inducing whole spawnings on a commercial scale still remain.

As the up-scaling of induction systems using chemical or heat-shock agents proved to be a significant challenge at the commercial scale, we also decided to investigate hydrostatic pressure as an alternative triploid induction agent for whole spawnings. Results from a CSIRO and Flinder's University, Seafood CRC PhD student project had demonstrated (at a small scale) that triploidy could be induced in Giant Tiger prawns using hydrostatic pressure (Andrew Foote, CSIRO Unpublished Information). A spawning tank sized pressure vessel would eliminate any requirement for handling of the fragile embryos prior to application of the shock agent and was therefore engineered and built by CSIRO for use in this this project (Appendix 1). Females successfully spawned in the chamber and spawnings were successfully induced to be triploid, with assessment made only to nauplii stage.

There is now substantial data and knowledge resulting from the R&D into Giant Tiger prawn triploid induction which is ready for consideration by commercial hatcheries for commercial scale optimisation. It must be noted however that this optimisation will take significant investment in hatchery staff time in order to develop procedures to suit commercial practices. Despite this, the knowledge and technology behind whole spawning triploid induction using a variety of techniques is now well documented and freely available to any Australian industry partner interested in trialling at their hatchery.

OUTPUTS ACHIEVED

1. Whole spawning triploid induction methodologies for Giant Tiger prawns:
- 90 uM mesh pen for temperature and chemical shock induction
- Spawning chamber sized pressure vessel for pressure shock induction

2. Knowledge of the growth and survival performance of tank-reared triploid Giant Tiger prawns compared to diploid siblings: There is no significant difference between the growth rates of triploid females and triploid and diploid males, however, diploid females grow slightly larger in the same time frame. Diploids had higher survival rates than triploids.

3. Knowledge of the sex ratio and reproductive sterility of a triploid (chemical shock only) Giant Tiger prawn family*:* Triploid sex ratio of 1 female: 1.625 males. Triploid females and males were incapable of producing viable offspring when mated with wild-caught diploids, and their diploid siblings were fertile. Cellular structure and organisation of triploid female and male gonads was severely compromised compared to diploid siblings at harvest and reproductive age. Triploidy would be a suitable means to confer genetic protection in Giant Tiger prawns provided the challenges of attaining a high hatch, survival and induction rate when inducing a whole spawning on a commercial scale are overcome.

4. A spawning tank sized pressure vessel was successfully tested (Appendix 1). Giant Tiger prawns successfully spawned within the pressure vessel and whole spawnings could be induced with pressure to produce triploids requiring no physical handling of the embryos during the first hour after spawning when they are extremely fragile.

5. Dissemination of triploid induction procedures to Australian industry members has occurred by conducting triploid inductions on-farm, along with completion of two scientific publications currently at external review:

- Sellars, M.J., Wood, A., Murphy, B., McCulloch, R.M., Preston, N.P. At review. Triploid Black Tiger shrimp (*Penaeus monodon*) performance from egg to harvest age. Aquaculture.

- Sellars, M.J., Wood, A., Murphy, B., Coman, G.J., Arnold, S.J., McCulloch, R.M., Preston, N.P. At review. Reproductive performance and mature gonad morphology of triploid and diploid Black Tiger shrimp (*Penaeus monodon*) siblings. Journal of Animal Science.

The knowledge and technology behind Giant Tiger prawn triploidy is now well documented and freely available to any Australian industry partner interested and willing to trial this procedure at their hatchery. Interested persons should contact CSIROs Food Futures Flagship or the Seafood CRC for more information. Melony.Sellars@csiro.au

1. Introduction

Domestication and selective breeding programs are currently progressing at several Australian farms. The most advanced of the selected lines developed in the breeding programs have demonstrated high commercial pond performance over several generations. Importantly, the broader distribution of these high performance lines to other grower companies poses a risk for the breeding program companies, through loss of their investment by potential on-sale or on-rearing of their selected post-larvae. One avenue to overcome broader distribution of the selected post-larvae, but without the risks to breeding companies of losing their genetic investment, is to have a fail-proof means of genetic protection.

Given the pond performance of these selected genotypes, the Australian prawn industry, represented by the Australian Prawn Farmers Association (APFA), has identified triploid prawn production as having the potential to provide a level of genetic protection of seedstock, based on evidence that triploid prawns were found reproductively sterile in those species previously studied.

Over the last 12 years CSIRO's FFF has been strategically investigating triploidy in penaeid prawn species with the vision that this technology may provide an avenue for the Australian industry to produce reproductively sterile prawn populations. At CSIRO we have developed triploid penaeid prawn induction protocols and demonstrated under laboratory and tank rearing conditions that triploid Japanese Tiger prawns, *Penaeus japonicus,* are reproductively sterile. Recently we adapted our protocols to allow heat shock (warm water) induction instead of chemical which is considered to be more acceptable to consumers and thus a more suitable option for commercialisation of this technology. Using our experimental systems we have been achieving triploid induction rates of up to 80%.

This project aimed to take CSIROs well optimised laboratory scale triploid induction methodologies and up-scale them to induce whole Giant Tiger prawn spawnings to be triploid using a technique that would be suitable for commercial up-scaling. Close industry engagement was sought throughout the project to allow practical solutions to complex experimental problems and dissemination of methodologies to commercial hatchery staff. The project also assessed the performance of triploid Giant Tiger prawns from egg to reproductive maturity for the first time.

1.1 Need

A mechanism to confer genetic protection of seedstock with elite genotypes would allow breeding companies to on-sell selected post-larvae to other companies without risk of losing their investments in developing these lines. Furthermore, such seedstock sale would provide an avenue for grower companies to access higher performance genotypes for their commercial pond production. To-date there is no commercial method for fail-proof genetic protection of prawns. Triploid induction is the only experimental methodology that shows promise as a means of genetic protection, however only laboratory-scale induction methods have been reported. For Black Tiger prawns, there are few reports of studies attempting triploidy induction, and certainly no studies have on-reared larvae through to harvest age or further to reproductive maturity.

Up-scaling of laboratory triploid induction methods to allow induction of whole spawns will be a necessity to allow commercialisation of this technology. There is a need to trial different methodologies for up-scaling the induction procedures using the shock agents which have been proven successful, whilst also assessing the impacts of up-scaling on hatching rate, survival, growth of stocks, reproductive performance and ploidy level.

Rigorous data on triploidy performance is only available for two penaeid prawn species, Chinese prawn (*Fenneropenaeus chinensis*), and Japanese Tiger prawn (*Penaeus japonicus*). In the present study, it was considered essential to conduct rigorous performance assessments of triploid Giant Tiger prawn so that the Australian industry can be confident that this technology will be suitable for genetic protection in this species.

1.2 Objectives

1. Develop *P. monodon* triploid heat shock induction systems that allow all eggs spawned from any one female to be exposed to heat shock at the same time.

2. Determine the performance (growth, survival, reproductive sterility and sex ratio) of triploid *P. monodon* when reared to reproductive maturity compared to diploids.

3. Develop commercially relevant *P. monodon* triploid induction protocols for the Australian industry.

4. Transfer commercially relevant triploid induction technologies to one or more commercial hatcheries.

2. Methods

2.1 Development of whole spawning induction methods using temperature and chemical shock agents

2.1.1 Broodstock source, conditioning and spawning

Wild-caught broodstock collected from a population off the coast of Innisfail (17°53'S, 146°01'E), Queensland, Australia were conditioned for spawning in 10 tonne sand-based tanks at CSIRO Marine and Atmospheric Research, Australia or Gold Coast Marine Aquaculture (GCMA), Australia using standard industry practices. Females that were ripe (ready to spawn) were selected based on gonad development determined by shining a torch beam through the dorsal exoskeleton (Tan-Fermin and Pudadera, 1989). Ripe females were transferred to 100 L flow-through circular spawning tanks filled to 60 L (water flow 0.7 L min^{-1}, water temperature 28.5°C, moderate aeration), and fitted with an automatic spawning detection device (Coman et al., 2003). Inductions were performed both at CSIRO and at a commercial hatchery depending on the source of broodstock.

The spawning detection device detects *P. monodon* embryos whilst still being released from the female. Time zero was taken as the time when the spawning detection device was triggered. For ease of explanation, spawned eggs, whether fertilized or unfertilized, will be referred to as embryos from hereon.

2.1.2 Whole spawn concentration methods for triploid induction

Several devices were designed in an attempt to concentrate embryos to allow application of the shock agent at the required time post-spawning detection (psd) (approx. 7 min 30 sec). Significant design input was attained from members of the Australian Industry through email and phone contact. There were four main designs trialled (Figure 1);

1) drainage outlet screens with increased surface area to reduce pressure on the embryos as the water volume was reduced and embryos were concentrated,

2) an egg concentrator that accumulated the majority of a spawn in the bottom 5 cm of tank water allowing the top layer of water to be siphoned off with large siphons whilst not putting any pressure on the embryos,

3) a larger tank volume and colander allowing large volumes of heated seawater to be added in a dispersed manner to result in the desired treatment temperature, and

4) a mesh pen design (initially 150 µM and later 90 µM) that was fitted in the spawning tank before the ready-to-spawn female was put in, allowing eggs to be concentrated as the pen was slowly lifted out of the tank.

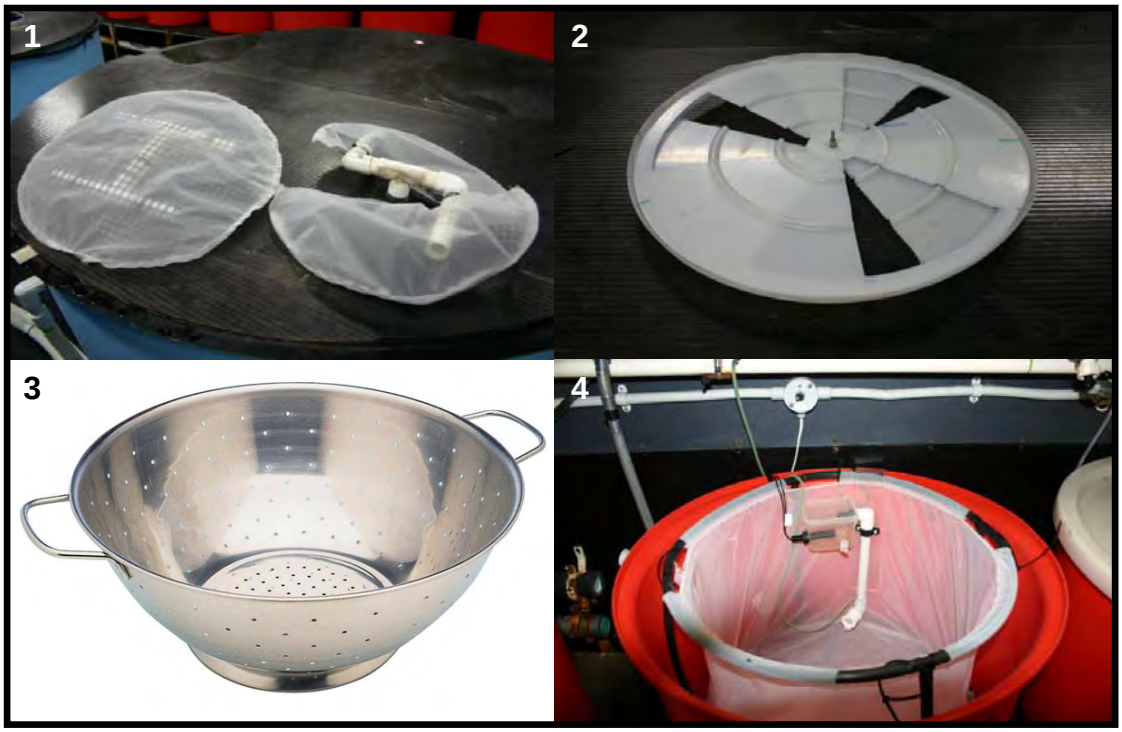

Figure 1. The four main devices trialled for concentrating embryos and applying the shock agents at the required 7 min 30 sec psd treatment time; 1) drainage outlet screens, 2) egg concentrator in the *open* position, 3) colander used in conjunction with larger tank, and 4) 90 μm mesh pen.

2.1.3 Triploid induction using drainage outlet screens and heat shock

On sounding of the spawning detection alarm the aeration and water intake were removed from the tank. Once the female ceased releasing eggs or at 2 min psd (whichever was first), the female was removed from the spawning tank; an 8 L volume of seawater and embryos was removed and set aside to become the control; a drainage outlet screen was installed and the water volume was dropped immediately to concentrate the embryos. At 7 min 30 sec psd the remaining tank volume was measured and the required quantity of 38°C seawater added gently to result in a treatment temperature of 34.8 to 35.3°C. The treatment duration ranged from 8 to 10 min. Once embryos had settled during the treatment phase, the surface water with no or few embryos was slowly siphoned from the tank to reduce the total tank volume to approximately 20-35 L of seawater and embryos. Once the treatment time had elapsed, 28.5°C water was added to cease the shock. The control 8 L of seawater and embryos was treated with similar physical stresses. Control and treatment embryos were given gentle aeration and maintained at 28.5°C until hatching 12-14 h psd. This induction procedure was repeated for 7 spawnings (i.e. 7 families).

2.1.4 Triploid induction using an egg concentrator and heat shock

At time of alarm the aeration and water intake were removed from the tank and a 5 cm high plastic ring submerged in the bottom of the tank. Once the female ceased

releasing eggs or at 2 min psd (whichever was first), the female was removed from the spawning tank and the egg concentrator device slowly submerged to the bottom of the tank with the device holes in the *open* position until reaching the plastic ring. The holes were then closed by rotating the second layer of the device and the above water volume removed by large 8 cm diameter siphons. At 7 min 30 sec psd a 250 mL volume of seawater and embryos was removed and set aside to become the control, the remaining tank volume measured and the required quantity of 38°C seawater added gently to result in a treatment temperature of 34.8 to 35.3°C. The treatment duration ranged from 8 to 10 min. Once the treatment time had elapsed, embryos were gently poured into a large volume of 28.5°C water to cease the shock. The control 250 mL of seawater and embryos was treated with similar physical stresses. Control and treatment embryos were given gentle aeration and maintained at 28.5°C until hatching 12-14 h psd. This induction procedure was repeated for 10 spawnings.

2.1.5 Triploid induction using a colander and heat shock

At time of alarm the aeration and water intake were removed from the tank and total tank volume measured. A 9 L bucket of seawater and embryos was taken from the spawning tank at 6 min psd and set aside to become the control. The total volume of 80°C seawater required to result in the treatment temperature of 34.8 to 35.3°C was calculated and at 7 min 30 sec psd poured through a colander to increase the surface area over which it was added and minimise embryo disturbance. The treatment duration ranged from 8 to 10 min. Once embryos had settled during the treatment phase, the surface water with no or few embryos was slowly siphoned from the tank to reduce the total tank volume to approximately 40 L of seawater and embryos. Once the treatment time had elapsed, embryos were gently poured into a large volume of 28.5°C water to cease the shock. The control 9 L of seawater and embryos was treated with similar physical stresses. Control and treatment embryos were given gentle aeration and maintained at 28.5°C until hatching 12-14 h psd. This induction procedure was repeated for 6 spawnings or families.

2.1.6 Triploid induction using a mesh pen and heat shock

A 150 or 90 μm mesh pen was fitted in the spawning tank prior to putting the ready-to-spawn female in the tank. At time of alarm the aeration and water intake were removed from the tank. Once the female ceased releasing eggs or at 2 min psd (whichever was first), the female was removed from the spawning tank. An 8-9 L bucket of seawater and embryos was taken from the spawning tank at 6 min psd and set aside as the control. At ~6 min psd the mesh pen was slowly lifted from the tank and embryos were gently rinsed down the side of the pen by hand so they remained in seawater. At 7 min 30 sec psd the pen was lifted from the 28.5°C spawning seawater and gently submerged in a treatment tank containing 34.5 to 37°C seawater. The treatment duration ranged from 8 to 10 min. At 1 min prior to the treatment time ceasing, the pen was slowly lifted upwards and embryos gently rinsed down the sides of the pen by hand. When the treatment duration time elapsed, the pen was lifted from the treatment seawater and gently submerged in a tank containing 28.5°C seawater to cease treatment. At 1 h psd the pen was gently lifted again and treated embryos were transferred to a commercial hatching system (Figure 2). The control 9 L of seawater and embryos was treated with similar physical stresses. Control and treatment embryos were given gentle aeration and maintained

at 28.5°C until hatching 12-14 h psd. This induction procedure was initially performed on 11 spawnings with a 150 µM mesh pen however embryos were disintegrating as they came into contact with the mesh and causing it to become clogged, preventing exchange of water. The procedure was then repeated for another 12 spawnings with a 90 µM mesh pen which allowed more rapid transfer of water, despite some embryo disintegration when they came into contact with the mesh.

As embryos were still disintegrating when coming into contact with the mesh, the induction procedure was again altered in an attempt to further minimise embryo damage whereby the spawned embryos and seawater were gently removed from the spawning tanks with 9 L buckets and placed in a 90 µM mesh pen already suspended in a 2-tonne volume of seawater to allow treatment at the desired temperature. After the treatment duration elapsed they were treated the same as done previously for other pen induced spawnings. This procedure was repeated for 22 spawnings.

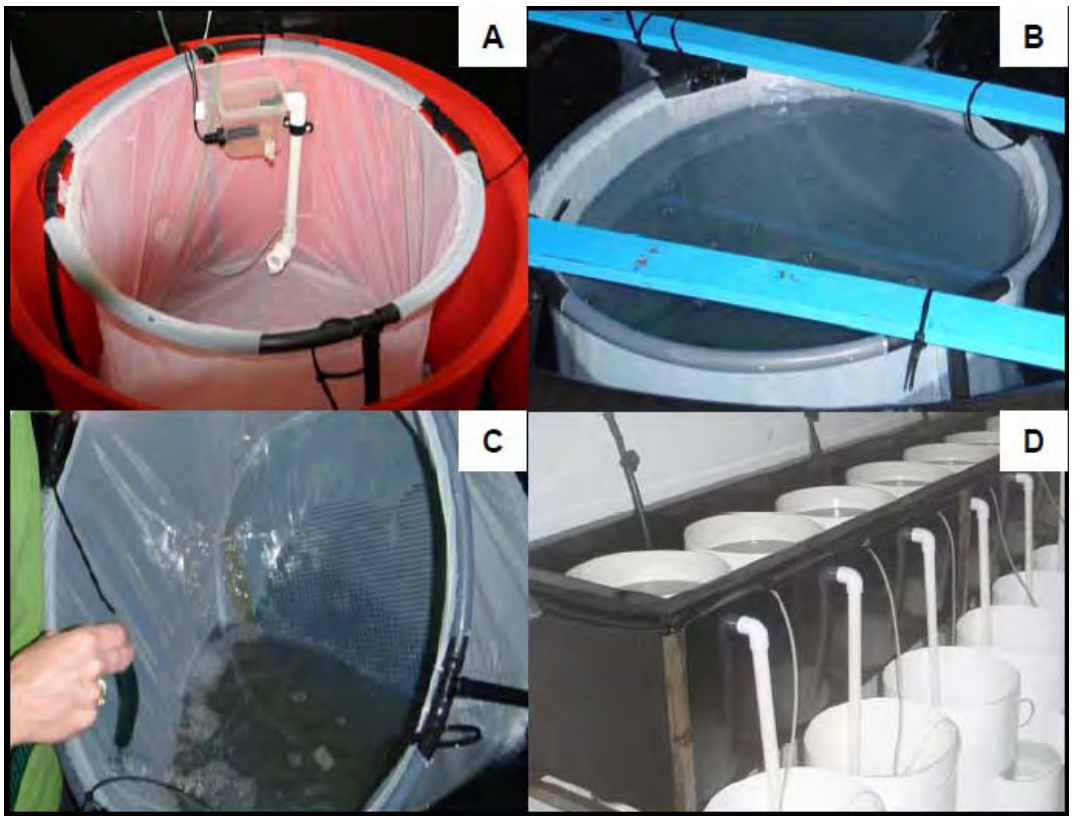

Figure 2. A) A 100 L spawning tank fitted with an automatic spawning detection device and an internal 90 µm mesh pen, B) The 90 µm mesh pen placed in a 1,000 L tank with seawater to cease treatment, C) Embryos being gently rinsed to the bottom of the pen at 1 h post-spawning prior to transfer into D) the commercial hatching system. Figure adapted from Sellars et al., 2011a in review, Figure 1.

2.1.7 Triploid induction using an adapted version of the 90 µM mesh pen and chemical shock

Chemical shock induction (using a final concentration of 200 µM 6-dimethylaminopurine or 6-DMAP, optimised from previous CSIRO research) was trialled on whole spawnings when using an adapted version of the 90 µM mesh pen

methodologies described above (this method showed the most promise in terms of minimal handling stress to embryos).

When using the 90 µM mesh pen method the pen was left in the spawning tank and at ~4 min post-spawning the female was removed along with all aeration and the spawning detection device. A control was taken as described when using the pen and heat shock. At 6 min 40 sec post-spawning 6-DMAP was added to the remaining 52 L to give a final treatment concentration of 200 µM. Gentle aeration was used immediately after addition of 6-DMAP for ~5 sec to ensure even mixing. The same methods for treatment duration, cessation and hatching of heat shock pen induced spawnings were used. This induction procedure was repeated for 14 spawnings or families.

2.1.8 Hatch rate assessments and unhatched egg and nauplii I ploidy analysis

Hatch rates of control and treatment embryos were assessed at 14-16 h psd either visually or by counting one to three 250 mL sub-samples as described previously (Sellars et al., 2011a). The method chosen for assessment was based on time available to do the assessment, whether or not any nauplii hatched and the numbers that were available for sampling and ploidy analysis.

Three replicates of 50 nauplii from each treatment and two replicates of 50 nauplii from the controls were sampled and snap-frozen on dry ice or in liquid nitrogen for ploidy analysis. In some instances three replicates of 50 unhatched embryos from each treatment and one sample of 50 unhatched embryos from the controls were sampled and snap-frozen on dry-ice or in liquid nitrogen for ploidy analysis. In instances where the commercial hatching system was used (Fig. 2D), three replicates of 50 nauplii were taken from the bottom chamber (which contains all hatched nauplii with a strong swimming behaviour) and two replicates of 50 nauplii from the controls were sampled and snap-frozen on dry ice or liquid nitrogen for ploidy analysis. Ploidy analysis was performed as described by Sellars et al., 2010 on a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter Australia Pty Ltd). A total of 50,000 prawn cells were analyzed per sample and the rate of ploidy determined by Quanta Analysis software (Beckman Coulter Australia Pty Ltd).

In one instance, a spawning induced on-farm to be triploid using the 90 µM mesh pen design and chemical shock was deemed suitable for commercial rearing based on high ploidy level of the nauplii and reasonable hatching rate. The family was reared from egg to reproductive age in a combination of commercial hatchery tanks and controlled environment sand-based tanks at CSIRO (section 2.3 Triploid Giant Tiger prawn performance from egg to reproductive age below).

2.2 Pressure treatment for whole spawning inductions

2.2.1 Engineering and specifications of the pressure chamber

As the up-scaling of laboratory induction systems to commercially suitable whole spawning induction systems whilst maintaining high hatch rates and induction rates

proved to be the key challenge of this project (to be discussed below), we decided to additionally investigate hydrostatic pressure as an alternative triploid induction agent for whole spawnings. Results from a CSIRO and Flinder's University, Seafood CRC PhD student project run in parallel demonstrated on a small scale that triploidy could be induced in Giant Tiger prawns using hydrostatic pressure (Andrew Foote, CSIRO Unpublished Information). A specialised spawning tank sized pressure vessel would eliminate any requirement for handling of the fragile embryos prior to application of the shock agent and was therefore engineered and built by CSIRO for use in this project (Appendix 1). Significant industry input was obtained throughout the engineering process via meetings, one-on-one farm visits, emails and phone conversations.

In brief the pressure chamber was designed to allow continual flow through seawater until the time of pressurisation. The total tank volume was 110 L and rated to 3,000 psi. The top of the pressure vessel had a smaller quick release end cap which allowed sealing of the chamber and pressurisation within the required time frame. The chamber was also fitted with a larval exit tube to allow nauplii harvesting as done in the commercial systems (Fig 2D). The chamber was engineered in a manner that would be fully upgradable to an automated triploid induction system.

2.2.2 Broodstock source, conditioning and spawning

Selected eighth (G8) generation broodstock *P. monodon* from GCMA were conditioned for spawning in 10 tonne sand-based tanks as part of the company's usual commercial practice. Ripe females were selected and transported to CSIRO Cleveland at a density of 3-4 females per styrofoam box (containing 25 L of 28.5°C seawater and a fish liner bag filled with oxygen); this was performed daily between 1600 to 1800 hours (~40 min drive). Upon arrival at CSIRO 3-4 females were placed in the spawning pressure chamber which was filled with 60 L seawater (water flow 0.7 L min^{-1}, water temperature 28.5°C, moderate aeration), and fitted with an automatic spawning detection device (Coman et al., 2003). On each night, remaining (extra) females were held in the same 100 L spawning tanks filled to 60 L as described above.

2.2.3 Triploid induction, harvesting embryos, hatch rate estimations and ploidy determination

At time of alarm the aeration was removed and the "Larval Exit Tube" plug fitted (Appendix 1), allowing the chamber to begin filling with seawater. Once the female ceased releasing eggs or at 2 min psd (whichever was first), all females were removed from the pressure tank and placed in 100 L holding tanks filled to 60 L. Once females were removed a 6 L sample of water and embryos was put aside as a control. The quick release end-cap was then fitted, and water inflow and end cap outflow valves were turned to the off position once all air was replaced with seawater in the vessel. At between 5 min 30 sec or 7 min 40 sec psd the chamber was pressurised to 1,500 or 2000psi taking approximately 2min to 2min 30 seconds to reach treatment pressure. The treatment duration was 10 min, with release of pressure (taking off of the shock agent) being effective instantly, having no lag time. After treatment, embryos were left in the vessel for 1 h after which time the entire content of the chamber (seawater and embryos) was gently removed with buckets and transferred to a commercial hatching system (Fig 1D) set up at CSIRO. Control

and treatment embryos were given gentle aeration and maintained at 28.5°C until hatching 12-14 h psd. This induction procedure was repeated for 9 spawnings.

Hatch rates were assessed and ploidy level of unhatched eggs and nauplii were determined as described in section '2.1.8 Hatch rate assessments and unhatched egg and nauplii I ploidy analysis'.

2.3 Triploid Giant Tiger prawn performance from egg to reproductive age

As detailed above, a spawning induced on-farm using the 90 µM mesh pen design and a chemical shock was deemed suitable for commercial rearing based on high ratio of triploid nauplii and reasonable hatching rate. The materials and methods for rearing this family from egg to harvest age are detailed in Sellars et al., 2011a whilst the materials and methods for this family's reproductive performance and mature gonad morphology assessments are detailed in Sellars et al., 2011b.

In brief, triploid induced nauplii were reared to PL10 at a commercial hatchery during which time their ploidy level was assessed at the different larval stages. At PL10 a sample of prawns had their dry weight and ploidy level determined, and all remaining PL10s were harvested using standard industry practices. The triploid induced family was subsequently reared from PL10 to PL184 (harvest age) in controlled environment clear-water tank systems at CSIRO. At PL28, PL60 and PL184 the number of prawns alive, weight and ploidy level of a sub-sample were determined. At PL184 prawns were also phenotypically sexed, the gonad histology of 12 of each of the four treatment classes assessed (diploid female, diploid male, triploid female and triploid male), and male reproductive tract, spermatophore and sperm quality assessed for diploids and triploids.

In total 20 G1 individuals from each of the four treatment classes (80 prawns in total) were individually eye-tagged and reared to reproductive maturity at CSIRO in clear-water tank systems. To undertake a rigorous reproductive performance assessment of the G1 triploid and diploid, female and male siblings, a combination of G0 wild crosses were performed when G1 prawns were PL315 (Table 1). A 55 day maturation conditioning period followed after which time a comprehensive 16 day reproductive performance trial was undertaken. During this time ovarian maturation and cycling, fecundity, hatch rate, gonad histology, thelycum impregnation, and male reproductive tract, spermatophore morphology and sperm quantity were assessed.

Table 1. Experimental crosses used and the number of prawns stocked when G1 siblings were PL315 and G0 prawns were collected from the wild. Tabled adapted from Sellars et al., 2011b.

	Females		Males	
Tank	Generation / ploidy	Number	Generation / ploidy	Number
1	G_0 diploid	20	G_1 diploid	20
2	G_0 diploid	18	G_1 triploid	18
3	G_1 diploid	11	G_0 diploid	29
	G_1 triploid	8		
	G_0 diploid	10		

4	G_1 diploid	11	G_0 diploid	29
	G_1 triploid	8		
	G_0 diploid	10		

3. Results

3.1 Development of whole spawning induction methods using temperature and chemical shock agents

3.1.1 Triploid induction using the drainage outlet screens and heat shock

Overall this induction method was not successful; resulting in high mortality rates of treated embryos from the 7 spawnings compared to controls (i.e. 5-8% hatching in the treatments compared to 65-70% in the controls). Embryos were being damaged and lost (essentially disintegrating when they came into contact with the mesh outlet screen) before the shock treatment was even applied. Triploids were present amongst those few nauplii that did hatch in the treatments however, as the impact on embryo survival from the draining method was so great, it is not possible to draw any meaningful conclusions on triploid induction from these 7 spawnings.

3.1.2 Triploid induction using the egg concentrator and heat shock

The egg concentrator functioned as anticipated, resulting in no physical stress being applied to those negatively buoyant embryos below the device when the top volume of water was rapidly removed by siphoning. However, after the device was submerged and then turned to the closed position, significant numbers of embryos were still present in the top water column (eg. 20-50% depending on the spawn) which was subsequently removed and dumped as part of the shock treatment process. Within the first 7 min post-spawning (or release from the female) *P. monodon* embryos were observed to be extremely buoyant as compared to other penaeid species like *Penaeus japonicus* and *Litopenaeus vannamei*.

Once the treatment shock was applied, it was noted that some embryo disintegration, as was also seen with the drainage outlet screens, was occurring however, in relatively smaller proportions. This was presumably from the physical stress of adding the heated seawater and embryos being tumbled around in the tank and hitting tank surfaces (disintegrating or exploding in the process as they are so fragile at this time). Hatch rates of treatment embryos from the ten spawnings were still significantly lower than for control embryos of the same spawns (22-31% hatching in the treatments compared to 65-70% in the controls). As for the previous 7 spawnings where the outlet screens were used, triploids were present amongst those nauplii that did hatch in the treatments however, as the impact on embryo survival from the physical stressors on the induction process were so great, it is not possible to draw any meaningful conclusions on triploid induction from these 10 spawnings.

Although the concentrator was a step in the right direction in terms of minimising physical stress to extremely immature and fragile embryos, it was not appropriate for treating 100% of embryos in a spawn, and the physical stress of applying the temperature shock was still beyond what the fragile embryos could withstand, thus preventing high hatching rates.

3.1.3 Triploid induction using the colander and heat shock

Overall this induction method was not successful; resulting in high mortality rates of treated embryos from the 6 spawnings compared to controls (i.e. 2-8% hatching in the treatments compared to 65-70% in the controls). Embryos were being damaged and lost (essentially disintegrating when they came into contact with the tank surfaces as they tumbled around) as the heated seawater was added to the tank to result in the desired shock temperature. Flow cytometry analysis was performed on those few nauplii that hatched however, as the impact on embryo survival from the addition of heated seawater was so great, no meaningful conclusions on triploid induction from these 6 spawnings can be made.

3.1.4 Triploid induction using the mesh pen and heat shock

The initial 11 spawnings which were induced using a 150 μm mesh pen resulted in extremely low or no hatch rates in the treatments compared to 60-70% hatch rates in the controls. This was a result of the embryos disintegrating as they came into contact with the mesh pen and water was pushing them against the mesh as part of the process of pulling the pen gently out the water. As a result 90 μm mesh pens were constructed and trialled on 12 more spawnings.

The 90 μm mesh pens significantly reduced the amount of embryo disintegration when the pen was pulled upwards out of the spawning tank however; hatch rates were still too low in the treatments (ave. 26.87 ± 0.05%) compared to controls (ave. 63.52 ± 0.09%) to warrant this as a suitable whole spawn induction method (Table 2). Triploid induction rates for these 11 spawns were highly variable but due to the high mortality rates the ploidy data from these spawns provides little insight into the induction procedures ability to induce triploids.

Given the problems with embryo disintegration as a result of being pushed against the mesh when water was moving through the pens when being lifted, it was decided to attempt gently bucketing the embryos into a pen already submerged in the treatment temperature water. This was completed on 11 spawnings and proved the most successful of all whole spawning induction methods trialled when combined with heat shock induction. Development rates to 2-cell of a sub-sample of embryos were calculated instead of hatching rates simply due to the logistics and timeframes of conducting inductions all night long at the commercial farm. Using this bucketing and 90 μm mesh pen method the development to 2-cell stage in the treatment was 78.2 ± 0.04% compared to 84.4 ± 0.03% in the controls (Table 2). As a result hatching rates were reasonably high in the treatments. Of these 11 spawnings, 2 resulted in no triploids being induced whilst the triploidy level of the other 9 spawnings ranged from 28.1 to 89.7% (Table 2). Despite reasonable numbers of triploid nauplii being produced using this method, simultaneously chemical shock inductions were also being performed and it was decided to rear on one of the chemical inducted triploid families (discussed below) which had a high triploid induction rate and high hatch rate. From a hatchery perspective it is easy to rear and manage larvae of the same age (i.e. spawned within 48 h of each other).

Overall the pen system was an improvement on previously trialled whole spawn induction methods, however the induction procedure needed fine tuning as it was very labour intensive and still resulted in some embryo mortality as a result of physical stressors during the induction procedure.

Table 2. Development to 2-cell, hatch rate and triploidy data for heat induced spawnings which used the 90 μm mesh pen when either lifted from the spawning tank or embryos and spawning water were gently bucketed into the pen already submerged in the treatment sweater.

			Development to 2-cell		Hatch rate		
Induction method	Spawning #	Induction temp. (°C)	Control	Treatment	Control	Treatment	Triploidy (nauplii only)
Pen lifted	1*	37			good	low	
	2	35.2			94.8%	47.8%	17.80%
	3	35.3			9.8%	14.5%	71%
	4	36.3			77.5%	28.0%	16%
	5	34.5			84.5%	38.7%	30%
	6	34.3			28.0%	24.6%	0%
	7	35.4			90.0%	32.0%	0%
	8	35.2			85.9%	51.8%	46% top, 33% bott
	9	34.9			64.0%	17.0%	61.8% top, 81% bott
	10*	34.7			51.4%	2.8%	
	11	34.8			49.3%	11.5%	100%
				Average	63.5%	26.9%	
				SE	0.09	0.05	
Embryos bucketed	1	34.6	93%	79%			51.6% top, 0% bott
	2	34.7	76%	55%			28.1% top, 0% bott
	3	34.5	78%	85.70%			0% top, 0% bott
	4	34.9	92.8%	85.70%			50.1% top, 86.8% bott
	5	35.4	80.4%	72.5%			0%
	6	35.4					65.6%
	7	35.4	82.4%	75.6%			70.0%
	8	35.1	72.8%	72.6%			83.5% top, 95.0% bott
	9	35.2	94.6%	95.2%			87.7% top, 0% bott
	10	35	97.7%	87.3%			89.7% top, 0% bott
	11	35	76.7%	73.2%			50% top, 0% bott
		Average	84.4%	78.2%			
		SE	0.03	0.04			

Development to 2-cell data was not collected for the 11 spawns in which the pen was lifted from the spawning tank.
Hatch rate data was not collected for the 11 spawns in which the embryos and seawater were bucketed into the pen.
* Treatment hatch rates were too low to warrant performing ploidy analysis of the nauplii.
top = nauplii sampled from the top vessel pictured in Figure 2D
bott = nauplii sampled from the bottom vessel pictured in Figure 2D

N.B. The hatch time of triploid nauplii may be longer than diploid nauplii and they may not be as strong. Because we typically took nauplii samples for ploidy analysis when we saw nauplii present, it wasn't always the case that there had been time for triploids to harvest into the bottom chamber.

3.1.5 Triploid induction using an adapted version of the 90 µM mesh pen and a chemical shock

Triploid induction using chemical shock in combination with the 90 µM mesh pen was successful with development to the 2-cell stage for the first three spawnings being 65, 53 and 42% in the treatments compared to 91, 46 and 40% in the controls (Table 3). Development data for the remaining 9 spawnings and hatch rate count data was not collected due to time restrictions with performing inductions at the commercial hatchery throughout the night (6pm to 4am) and returning within 12 hours of the first spawn to sample and perform ploidy analysis for both chemical and heat induction spawnings. Triploidy induction rates for these 12 spawnings averaged 83 ± 4% when nauplii were sampled from the top treatment hatching chamber (Figure 2D); this being where the majority of nauplii were collected from for flow cytometry analysis.

One chemical induced spawn (Spawning 10; Table 3) had suitably high nauplii numbers and triploid induction rate to be reared through at the commercial hatchery. This spawning /family had a nauplii hatching rate of ~65%; a triploidy induction rate of ~83%, as measured from three pooled nauplii samples; and a high larval quality based on visually observation of photo tactic response of nauplii to light. In total 183,000 nauplii from this triploid family were stocked in one 10-tonne parabolic tank at GCMA and reared according to commercial rearing practices through to post-larval (PL) stage 10. Subsequently, performance assessments were performed using this family.

Table 3. Development to 2-cell and triploidy data for chemical induced spawnings when using the 90 µM mesh pen.

	Development to 2-cell		Triploidy (nauplii only)	
Spawning #	Control	Treatment	Top	Bottom
1	91%	65%	91%	0%
2	46%	53%	96%	94%
3	40%	42%	76%	97%
4			96%	24%
5			84%	0%
6			90%	0%
7			54%	16%
8			89%	14%
9			76%	0%
10*			87%	79%
11			86%	0%
12			69%	0%
		Average	83%	27%
		SE	4%	11%

Development to 2-cell data was only collected for spawnings 1, 2 and 3.
Top = nauplii sampled from the top vessel pictured in Figure 2D
Bottom = nauplii sampled from the bottom vessel pictured in Figure 2D
* Triploid family that was reared for performance assessments.

N.B. The hatch time of triploid nauplii may be longer than diploid nauplii and they may not be as strong. Because we took nauplii samples for ploidy analysis when we first saw that there were nauplii hatched, it wasn't always the case that there had been time for triploids to harvest into the bottom chamber. In most instances the majority of nauplii in the treatments were still in the top chamber at time of ploidy sampling.

3.2 Pressure treatment for whole spawning inductions

A spawning chamber sized pressure vessel was constructed by CSIRO for use in the 2010 spawning season. Inductions were performed over several weeks using G8 *P. monodon* from GCMA. Initial changes to the aeration design of the vessel were required to allow prawns to spawn in the vessel; these included removal of physical impediments (i.e. removal of some bottom positioned aeration structures that were in the initial design).

Despite there being 9 successful spawnings in the chamber, only 3 spawnings produced any nauplii (i.e. 6 spawns had zero development to the 2-cell stage and thus no hatched nauplii). In these 3 spawnings treatment hatch rates were extremely low (1.7 to 9.4%; Table 4), and thus little can be drawn from the triploid induction rates which ranged from 2.9 to 57.7%.

These low hatch rates of the G8 *P. monodon* were likely an artefact of low fertilisation rates in the domesticated *P. monodon* used. This was supported by the observation that spare females spawning in the 100 L holding tanks also had poor embryo development and hatch rates – often zero (i.e. it was not a factor caused by the chamber). Subsequent spawnings in the chamber using wild *P. monodon* broodstock resulted in successful spawns with high hatch rates, up to 80.1%.

Ideally the pressure chamber would be fitted out with an auxillary pump that allows 2000 psi to be reached within 1 min as opposed to 2 min 30 sec. More rapid onset of shock application would better mimic the small scale experiments where we have demonstrated successful triploidy induction in *P. monodon* (i.e. CSIRO, Flinder's University PhD project of Andrew Foote - funded by the CRC). Furthermore, future large pressure chamber inductions using broodstock known to be highly fecund and fertile would provide more meaningful results in a short time-frame. The large pressure chamber, built by CSIRO Marine and Atmospheric Research, is available for use by the Australian Prawn Farming Industry if any member would like to finish the optimisation process. Significant OHS&E requirements do however need to be overcome prior to transfer of this instrument from CSIRO as it is still considered an experimental unit.

Table 4. Hatch rates and ploidy levels of nauplii from pressure induced spawnings that produced viable nauplii.

Hatch rate		
Control	Treatment	Ploidy (nauplii only)
25.5%	9.4%	2.9%
low	1.7%	57.7%
low	2.6%	24.6%

3.3 Triploid Giant Tiger prawn performance from egg to reproductive age

Detailed results for rearing this family from egg to harvest age are in Sellars et al., 2011a and for this family's reproductive performance and mature gonad morphology are in Sellars et al., 2011b. Below is a summary of the key results as presented in these two scientific manuscripts.

3.3.1 Hatching, triploid induction rates and total harvested nauplii from the triploid induced family

The hatch rate of the untreated control was estimated at 78%, which compared to an estimated hatch rate of 65% for the treated prawns. Control nauplii were 100% diploid and treatment nauplii were 83.40 ± 0.04% triploid. In total 183,000 nauplii were estimated to be harvested from the triploid induced family.

3.3.2 Triploidy rate, survival, weight and sex ratio throughout development to PL184

The triploidy rate of the triploid induced family from protozoea to PL60 ranged from 60.47 to 72.38% (Fig. 3). An additional observation that resulted from the triploidy analysis of the larval stages was that triploids took longer (approximately 12-24 hours) than diploids to metamorphose through each larval stage. The total triploidy rate (not separated for sex) from PL60 to PL184 showed a decrease from 72.38 to 56.32% (Fig. 3). Of the 261 PL184 prawns harvested, there were 56 triploid females, 65 diploid females, 91 triploid males and 49 diploid males (i.e. 1.14 triploid females: 1.33 diploid females: 1.86 triploid males: 1 diploid male) (Fig. 4).

Survival from PL28-PL60 in both the 100 L tanks and the 1,000 L tank ranged from 65 to 98% but overall was highest in the 1,000 L tank. Overall survival from PL28-PL60 was 70.5% and from PL60-PL184 was 54.0%.

At PL10 the estimated (from dry weight) mean wet weight per prawn was 4.91 mg, At PL28 the estimated wet weight per prawn was 46.00 mg from the 100 L tank rearing system and 66.00 mg from the 1,000 L tank rearing system. At PL60 the mean wet weight per prawn was 860.00 ± 0.45 mg.

At PL184, harvest age, diploid females were significantly larger (42.95 ± 2.43 g; *P* <0.001) than triploid females (35.14 ± 2.26 g; Fig. 5). Diploid males were, however, not significantly larger (34.02 ± 0.89 g; *P* >0.05) than triploid males (31.79 ± 1.56 g; Fig. 5). There was also no significant difference between the final harvest weight of triploid females and triploid and diploid males (*P* >0.05; Fig. 5).

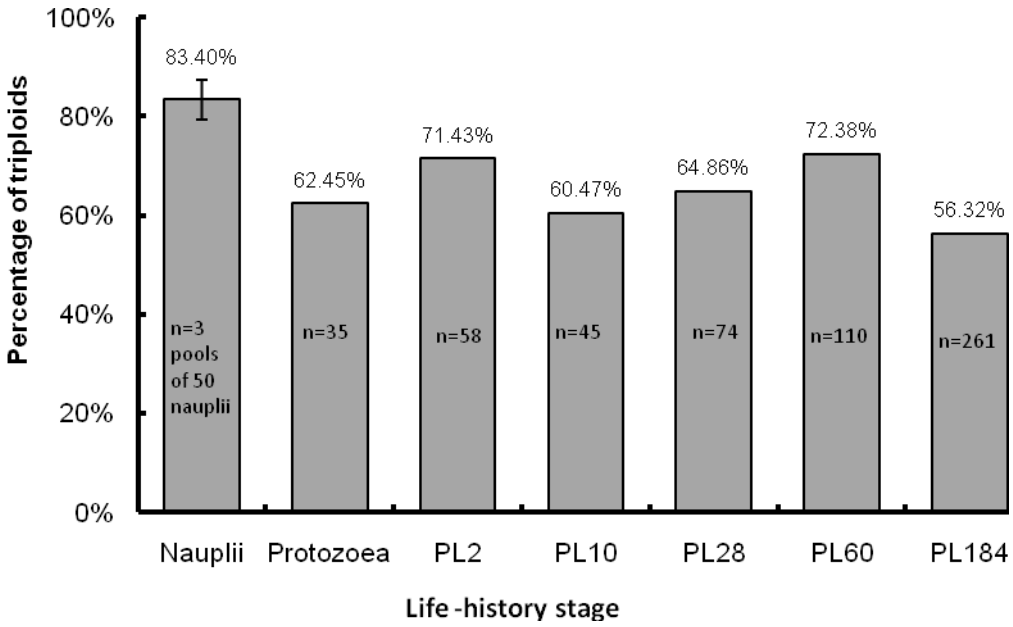

Figure 3. Triploidy rate of the triploid induced family throughout development and the number (n) of pools or individuals analysed. Samples were pooled for analysis of nauplii only and therefore S.E bars are only presented for this life-history stage. Ploidy of all other life-history stages was measured on individual prawns. Triploidy data presented was not separated for males and females at any of these life-history stages. Figure adapted from Sellars et al., 2011a.

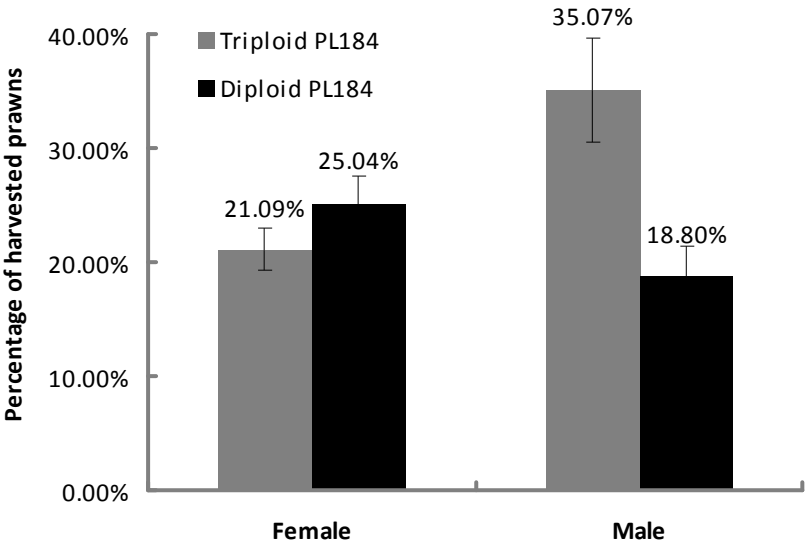

Figure 4. Triploidy rate and sex of harvest age PL184 ± S.E.. Percentages are calculated for the total population harvested, n=261. Standard errors are calculated based on the numbers of each category per tank. Figure adapted from Sellars et al., 2011a

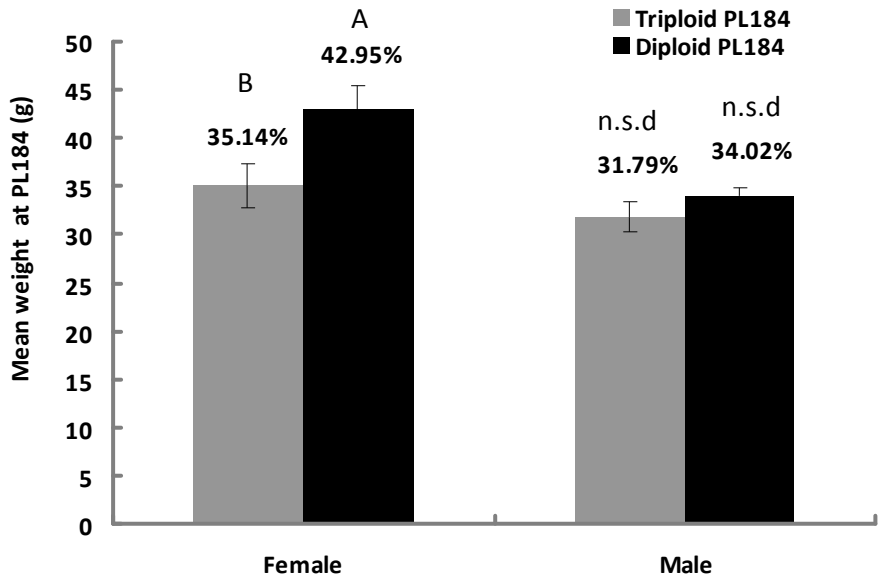

Figure 5. Mean weight of PL184, harvest age, triploid and diploid females and males ± S.E. from the triploid family. ANOVA and a least significance difference test of female weight data showed a significant difference between the mean weights as shown by uppercase letters at *P* <0.001. ANOVA of male weight data showed no significant difference between the different ploidy rates (n.s.d) at *P* <0.05. Figure adapted from Sellars et al., 2011a

3.3.3 Histological gonad and male reproductive tract assessment at PL184

Atypical ovaries were present in all 12 triploid PL184 females examined histologically. An acute reduction of developing oocytes was prominent in all triploid females compared to diploid females. No mature ova were present within any triploid female gonads examined. In comparison, no gonad abnormalities were observed in the 12 diploid female siblings examined; these females all having normal ovarian development with zones of proliferation and developing oocytes. As these diploid females were from the same treated cohort, results deompnstrate that the chemical treatment itself did not influence maturation; it is the ploidy status that has resulted in the difference.

Gross abnormalities were observed in all 12 triploid PL184 males examined histologically. In general, seminiferous tubules contained undifferentiated spermatocytes. Developing spermatogonia were not discernible at tubule periphery and a distinct lack of spermatozoa was noted within the lumens of the vas deferens. In comparison, no major gonad abnormalities were observed in the 12 diploid male siblings examined with seminiferous tubules containing normal zones of proliferation and mature spermatozoa present within the lumens of the vas deferens.

Spermatophores of triploid males were generally less visible during gross morphological observations. No sperm bundles were observed within the vas deferens and spermatophores of triploid males, whereas they were clearly visible within diploid males. The spermatosomatic index (spermatophore weight (g)/prawn weight (g) x 100) was significantly greater in diploid males than triploid males

(*P* <0.001) (Fig. 6) and counts of sperm from ejaculated spermatophores found that spermatophores from diploid prawns contained $8.18x10^6 \pm 1.16x10^6$ sperm cells while those from triploids contained no sperm.

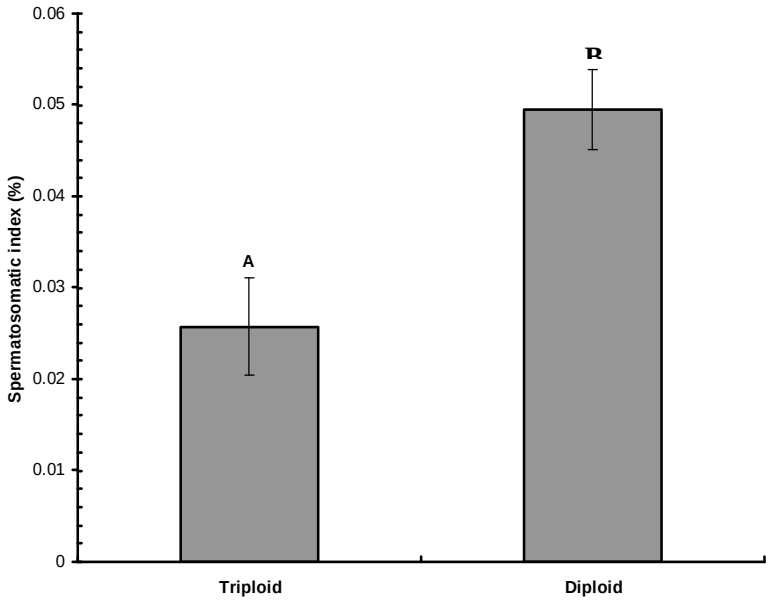

Figure 6. Spermatosomatic index (SSI) of triploid and diploid PL184 males, calculated by determining the percentage of ejaculated spermatophore weight to total prawn weight. Bars with different superscripts are significantly different (*P* <0.001). Figure adapted from Sellars et al., 2011a Figure 9.

3.3.4 Ovarian development, cycling and spawning frequency

After ablation, ovarian development and ovarian cycling (stage 0 to stage 4) between G0 diploid and G1 diploid females was not significantly different, whilst G1 triploid females failed to show any signs of ovarian development and cycling, remaining at ovary stage 0 throughout the trial. As a result there were no G1 triploid female spawnings. When assessing first spawnings only, there were 10 G0 diploid female x G0 male diploid spawnings and 9 G0 diploid female x G1 diploid male spawnings, all of which produced viable nauplii. In comparison there were 7 G0 diploid female x G1 triploid male spawnings, none of which produced viable nauplii. Despite the ovarian cycling in the G1 diploids being comparable to that of their wild counterparts, only a single spawning was achieved in the crossing with G0 males, and this was not fertile.

3.3.5 Reproductive performance of females and males

There were no significant differences (P >0.05) between the weights of females in the different treatment categories at time of ablation, and no difference in the weight of those which spawned (Table 5). All crosses with diploid females, whether G0 or G1 in origin, produced spawnings and thus eggs. Of those crosses which had more than one spawning (i.e. excluding the G1 diploid x G0 diploid cross which only had one spawn), nauplii were only produced when the diploid female was crossed with a

diploid male, regardless of whether G0 or G1 in origin (Table 5). No nauplii were produced from diploid female spawnings when crossed with G1 triploid males. As mentioned above, there were no G1 triploid female spawnings as triploid females did not produce mature gonads. Overall the performance of the G0 diploid x G1 diploid cross and the G0 diploid x G0 diploid cross was similar, whilst the G0 diploid x G1 triploid cross and the G1 triploid x G0 diploid cross did not produce any viable progeny.

When comparing the effects of the different male treatments (G0 diploid, G1 diploid or G1 triploid) on G0 female spawnings, there was no significant difference ($P > 0.05$) between the fecundity, percentage of spawnings that hatched and hatch rates of G0 diploid male and G1 diploid male spawns. The percentage of spawnings that were fertilized that hatched and hatch rates were however significantly lower ($P < 0.05$) for G1 triploid male spawnings when compared to G0 and G1 diploid male spawnings.

Table 5. Reproductive performance (±SE) of the different triploid and diploid *P. monodon* crosses, excluding the G1 triploid x G0 diploid (female x male) cross for which there were no spawnings. Table adapted from Sellars et al., 2011b.

	Cross (female x male)			
Measure	**G0 diploid x G1 diploid**	**G0 diploid x G1 triploid**	**G1 diploid x G0 diploid**	**G0 diploid x G0 diploid**
Female weight at 1st spawning	141.50 (8.31) *n=5* nsd	140.33 (3.74) *n=6* nsd	142.41 *n=1* *	143.49 (4.79) *n=10* nsd
Eggs (000's per spawning)	152.34 (8.9) *n=9* nsd	166.10 (21.9) *n=7* nsd	10.45 *n=1* *	159.14 (14.04) *n=10* nsd
Nauplii (000's per spawning)	77.96 (9.97) *n=9* B	0 *n=7* C	0 *n=1* *	57.96 (11.37) *n=10* A
% hatch per spawning	45.65 (7.65) *n=9* A	0 *n=7* B	na	33.02 (7.77) *n=10)* A
% spawnings that hatched	100 *n=9* A	0 *n=7* B	0 *n=1* *	100 *n=10* A
% embryo development per spawning	64.32 (9.92) *n=9* A	0 *n=7* B	na	46.20 (10.76) *n=9* A

n = number of events averaged
na = not applicable
* = excluded from ANOVA as only one replicate spawning
nsd = no significant difference (*P* >0.05)
Means with different uppercase letters are significantly different (*P* <0.05) within the 'Measure' parameter (rows)

3.3.6 Histological gonad morphology

Histological assessment of the three G1 triploid female gonads revealed that the number of developing oocytes was severely reduced. Few developing basophilic-staining pre-vitellogenic oocytes and eosinophilic-staining vitellogenic mature ova were observed within ovarian nodules (Fig 7a). G1 triploid females had few discernable nodules with typical structure. Nodule periphery connectives, follicle cells, germinal layer and haemal sinuses appeared normal; however the nodules were void of oogonia and contained only loose connective tissue. Oogonia appeared abnormal with enlarged nuclei containing fragmented chromatin and cells were boarded by loose connective tissue. Haemal sinuses were present but only surrounded limited numbers of oocytes compared to large numbers of developing oocytes and mature ova in diploid sibling controls (Fig. 7b). In the age-matched sibling comparison, the three G1 diploid females, and control comparison, the three G0 diploid females, normal ovaries with prolific numbers of developing oocytes and mature ova within the ovarian nodules were observed along with normal cellular organization (Fig. 7c). Overall, the development of the ovary was grossly impeded in all triploid G1 females examined whilst G1 and G0 diploid females had normal ovary morphology. The lack of developing oocytes and absence of rod like bodies in mature ova of triploids indicates normal ovarian maturation has been interrupted. Full maturity in G1 triploid females may never be achieved and the histology indicates that these females are reproductively sterile.

Histological assessment of the three G1 triploid males revealed abnormalities in the gonads of all examined prawns. A distinct lack of sperm cells within primary and secondary lumens of the vas deferens was observed in all cases (Fig. 8a). Spermatozoa present in the testes lacked the rigid spike required to initiate fertilization as described in triploid Penaeus chinensis by (Xiang et al., 2006). A lack of developing spermatagonia, mature spermatozoa and the absence of rigid spikes indicate complete sperm maturation has not occurred and indicates that the G1 triploid males will be reproductively sterile. In the age matched sibling comparison, the three G1 diploid males, and case control comparison, the three G0 diploid males, normal gonads with prolific numbers sperm cells within the primary and secondary lumens of the vas deferens and normal seminiferous tubules with cellular differentiation (Fig. 8b) were observed.

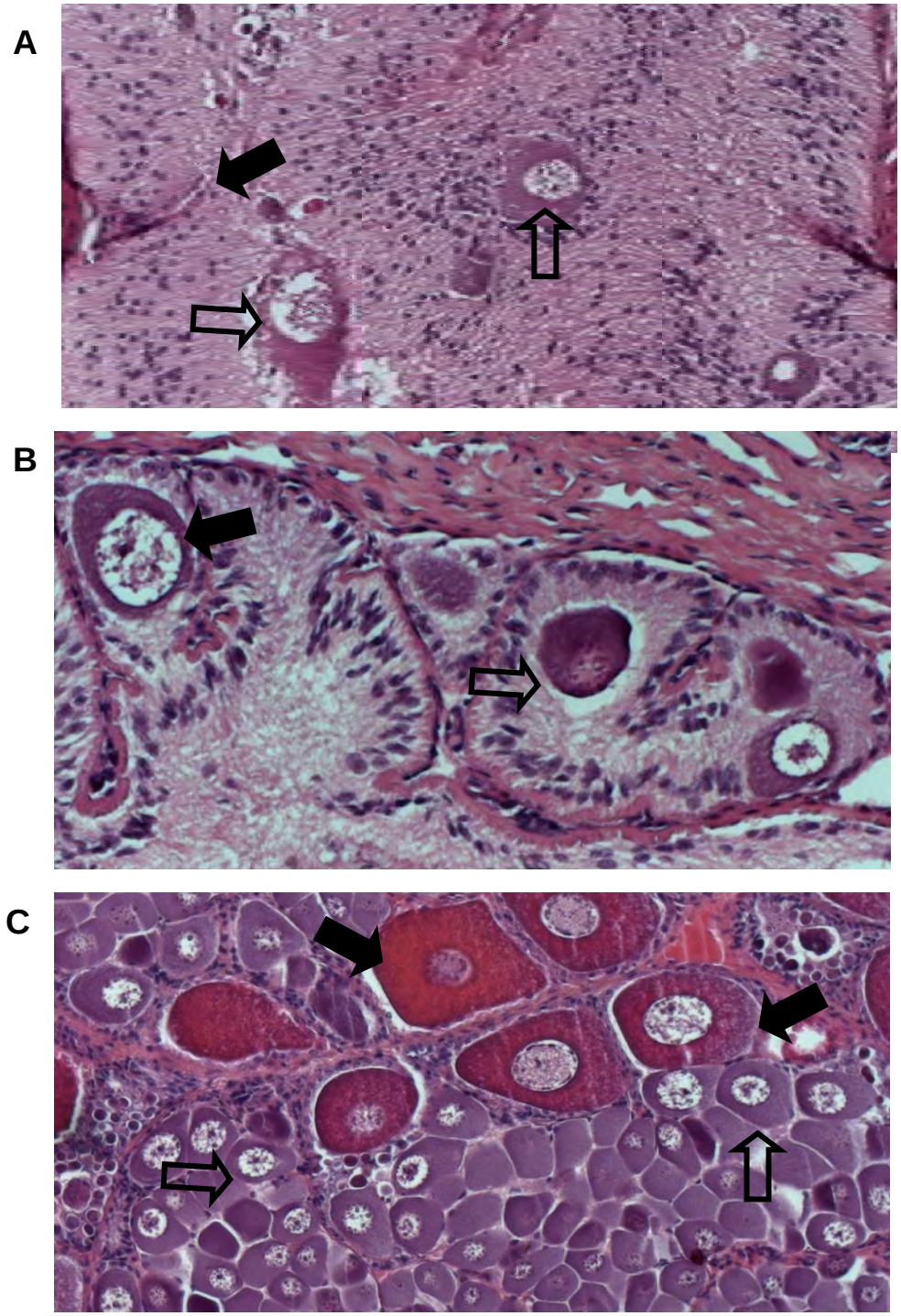

Figure 7. Haemotoxylin and eosin stained sections (10X magnification) of A) a triploid PL386 female gonad showing a complete reduction of developing oocytes (open arrows) and incomplete haemal sinuses (shaded arrow), B) a triploid PL386 ovary showing few discernable nodules with typical structure, loose connective tissue (open arrow) and few developing oocytes (shaded arrow), and; C) a sibling diploid PL386 female gonad showing normal ovarian morphology with prolific numbers of developing oocytes (open arrows) and mature ova (shaded arrows) within the ovarian nodules. Figure adapted from Sellars et al., 2011b Figure 1.

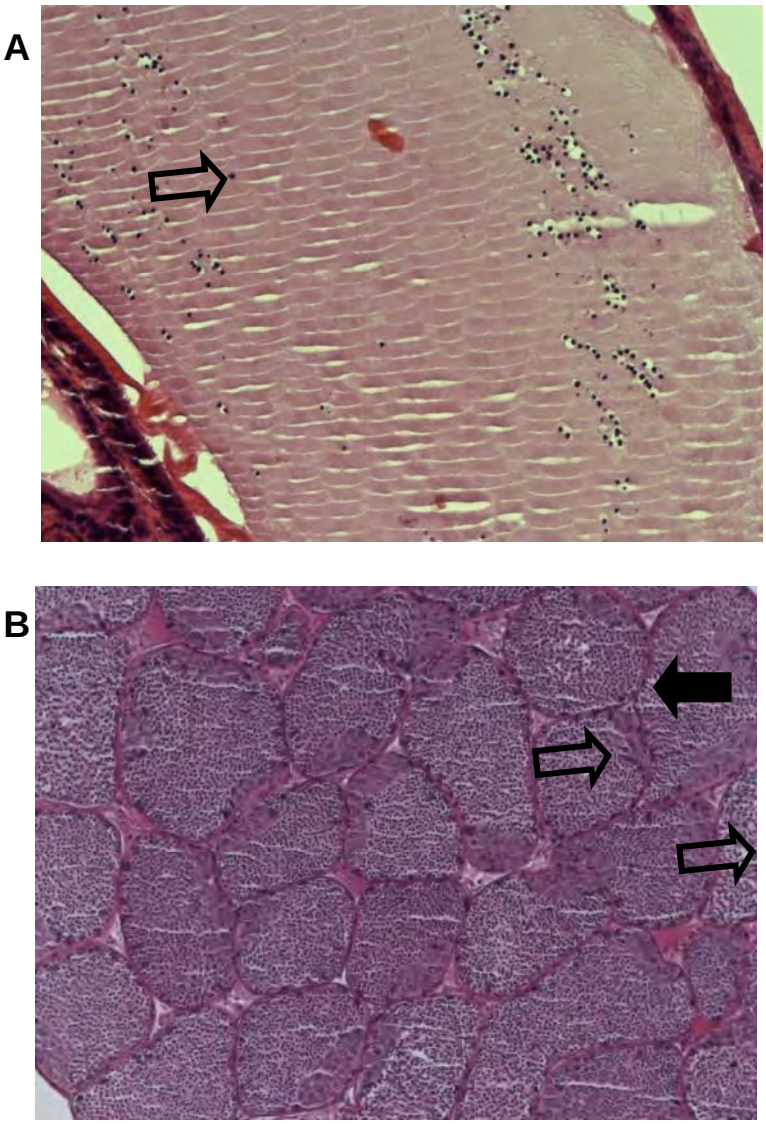

Figure 8. Haemotoxylin and eosin stained sections of A) a triploid PL386 male vas deferens (10X magnification) showing a distinct lack of sperm cells (open arrow); B) sibling diploid PL386 male seminiferous tubules (10X magnification) showing normal cellular differentiation with spermatogonia (open arrows), spermatozoa (shaded arrows) and haemal sinuses. Figure adapted from Sellars et al., 2011b Figure 2.

3.3.7 Male reproductive tract assessment and wild female x triploid male thelycum assessment

During external observations with the naked eye, spermatophores of G1 triploid males were only partially visible, while spermatophores of G0 and G1 diploid males were mostly fully visible. No sperm bundles were observed within the vas deferens and spermatophores of triploid males, whereas they were clearly visible within diploid males (Fig. 9). Spermatophores of G0 and G1 diploid males contained abundant sperm with normal morphology while those from G1 triploid males contained no sperm. The thelycum of 16 wild G0 diploid females that were crossed with G1 triploid males had no visible spermatophore nor sperm present when destructively examined (i.e. spermatophore remnants were examined within the thelyca dissected from the females).

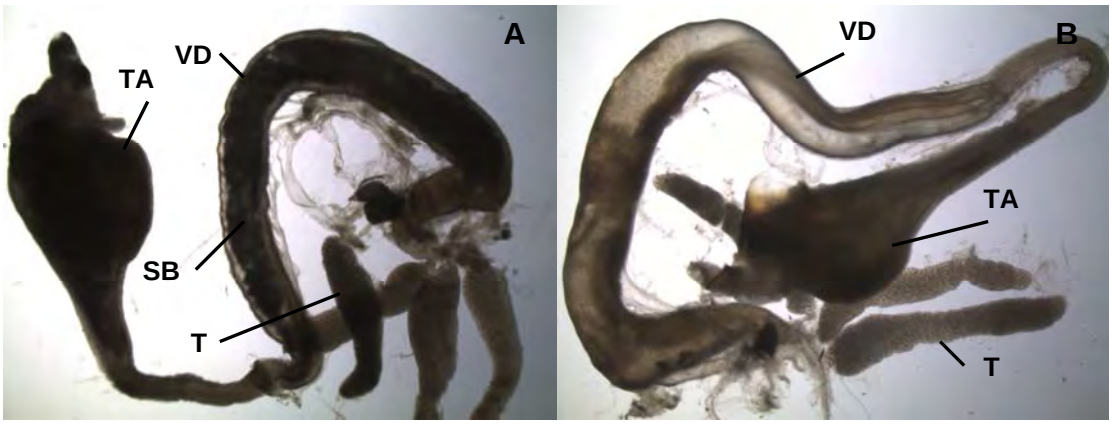

Figure 9. Reproductive tract from a PL386 male A) G1 diploid and B) G1 triploid showing a lack of sperm bundles in the triploid. VD = vas deferens, TA = terminal ampule, T = testes, SB = sperm bundles. Figure adapted from Sellars et al., 2011b Figure 3.

4. Discussion

4.1 Development of a whole spawning triploid induction system.

Up-scaling of experimental triploid induction systems to induce whole *P. monodon* spawns to be triploid proved to be the key challenge of this project. Despite being able to reliably induce triploidy, at high induction levels, under experimental conditions using various shock agents the biology (fragile nature) of newly spawned embryos made it extremely difficult to allow application of the shock agents to whole spawnings.

Based on industry and laboratory experience we estimate the minimum water volume required for a successful fertilised *P. monodon* spawning (i.e. for females of body weight of ~120 g) to be around 60 L of seawater. Any less than this and females do not spawn and/or fertilisation rates are compromised. As a result, the shock agents either need to be applied to a 60 L water volume or embryos need to be concentrated before 7 min 30 sec post-spawning when the shock agent needs to be applied.

Application of suitable quantities of heated seawater to make a 60 L volume of 28.5°C spawning water ~35°C for treatment by 7 min 30 sec post-spawning results in severe physical stress of the embryos as they interact with tank surfaces and essentially explode. Likewise, concentration of embryos to allow addition of a smaller volume of heated seawater to reach the desired treatment temperature results in embryos interacting with mesh surfaces as water volumes are reduced, making embryos disintegrate from the pressure against the mesh. Addition of boiling seawater to the 60 L volume essentially cooks many of the eggs, as the different temperature waters do not instantly mix. As the heat shock must be applied or put on within a short time-frame, other options for heating were also considered unsuitable.

Application of suitable quantities of chemical to the 60 L of seawater and embryos to induce triploidy was found a successful technique. However, chemicals are extremely expensive and quite toxic (i.e for handling by the user). Furthermore, after application of the shock agent at 8-10 min, the embryos are still moderately fragile and do not cope well with the physical pressures of removing the treatment. Despite these shortfalls, a triploid induced spawning using this induction method was reared to reproductive maturity and performance assessments were completed.

After completion of pressure scoping study to induce triploidy in *P. monodon* using small induction chamber (i.e. Andrew Foote's research), it was decided that a large spawning chamber size pressure vessel may provide the solution to whole spawning inductions.

A large pressure chamber was designed and constructed by CSIRO allowing prawns to successfully spawn and fertilise their embryos. Unfortunately during this project triploidy inductions with the large pressure vessel were only performed using G8 selected stocks, which had atypically poor fertilisation rates. The decision by industry to use these G8 stocks was made as such selected lines are the ultimate intended stocks for use of the induction system. In hind sight, this project would have benefited by trialling the large pressure chamber on broodstock females known to be highly fecund and fertile. Furthermore, ideally the pressure chamber would be fitted out with

an auxiliary pump that allows 2000 psi to be reached within 1 minutes as opposed to 2 min 30 sec as this would better mimic the small scale experiments demonstrated to be successful at inducing triploid *P. monodon.*

4.2 Triploid performance from egg to harvest age.

The ploidy, survival, weight, sex ratio and gonad morphology over time of sibling *P. monodon* triploids and diploids reared from egg to harvest age (PL184) are reported on in detail in Sellars et al., 2011a. Below is a brief summary discussion of the main findings.

4.2.1 Triploid rate throughout development and metamorphosis

The rate of triploidy gradually reduced as *P. monodon* progressed through the life-history stages from nauplii with 83.4% triploidy to PL184 (harvest age) when the triploidy rate dropped to 56.32%. This reduction in ploidy level has been noted in other penaeid species (Xiang et al., 2006), and likely also contributed by an artefact of the continual performance parameter measuring, and clear water tank rearing approach, used in this experiment.

Triploid *P. monodon* took longer (approximately 12-24 hours) than treated diploids to metamorphose through each larval stage in this chemical induced triploid family. Similar delays in metamorphsis rates have also been reported for heat induced triploid *F. chinensis* families with a similar delay time of ~24 hours (Xiang et al., 2006). A possible explanation for this time delay is that triploids have an extra set of chromosomes which may take longer to replicate at every cellular division, slowing development and resulting in the observed time delays in metamorphosing to the subsequent larval stage.

To be commercially viable, Australian producers of the selectively bred genotypes currently say that triploidy rates at harvest age would need to be in the range of 80-100% to be viable (Australian Prawn Farmers Association Pers. Com.). To achieve this, further optimization of the induction and rearing procedures would be required. Confirmation of the commercial suitability of the induced stocks would best be performed under commercial conditions (ponds) where prawns are not required to be removed from the rearing system for performance measurements throughout grow-out (i.e. through sacrificial sampling of the ponds).

4.2.2 Weight and sex ratio of triploid and diploid siblings throughout development and sex ratios

Weights throughout the performance assessment (i.e. from ages PL10 to PL60 and from PL60 to PL184) were similar to previous studies on G_1 *P. monodon* reared under the same clear-water tank systems with similar densities and diets (CSIRO unpublished information). At PL184 treated triploid females were significantly smaller than the treated diploid females. Similar studies on *M. japonicus* reported that once they reached PL173, treated triploid females are significantly smaller than untreated diploid females but are only generally smaller than treated diploid females (Coman et al., 2008). Although weight data separated by sex is not available for *F. chinensis*, reported triploidy growth rates are slightly different to *P. monodon* and *M. japonicus*, with triploids growing at a comparable rate to diploid siblings during the juvenile

stages (Xiang et al., 1998), but having better growth rates by the time they reach 6 months of age, (Xiang et al., 2006) which is an equivalent age to the PL184 *P. monodon* in this study and the PL173 *M. japonicus* in the study by Coman et al., 2008. In contrast to the females, at PL184 *P. monodon* diploid males were not significantly larger than triploid males. In this study there was also no significant difference between the final harvest weight of triploid females and triploid and diploid males. Overall, treated diploid female *P. monodon* grew faster than treated triploid siblings and treated diploid males in this study.

In this study the sex ratio of triploid *P. monodon* was about 1 female: 1.6 males. The high proportion of triploid male *P. monodon* in the present study was not expected. In comparison, triploid *M. japonicus* produced by stopping the same meiotic division using chemical shock are always female (Coman et al., 2008), whilst triploid *F. chinensis* produced by stopping the same meiotic division with heat or chemical shock have a sex ratio of 4 females: 1 male (Li et al., 2003; Xiang et al., 2006). Based on the results of this triploid family, triploidy in *P. monodon* does not confer predominantly female populations as for *M. japonicus* and *F. chinensis*, indicating that triploidy will not provide a suitable mechanism to skew sex ratios towards the faster growing females. Of note, the information on sex ratios found in this study has significant implications for scientific theory on how sex is determined in penaeid prawns.

4.2.3 Gonad morphology

Overall, a drastic reduction in the development of both ova and sperm in triploid prawns in comparison to diploids was observed. The limited numbers (and sometimes absence) of oocytes and sperm that were produced in the triploids failed to show signs of full maturation when examined histologically; suggesting that both triploid male and female P. monodon are reproductively sterile. Similar results have been reported for *F. chinensis* where triploids have a drastic reduction in both ova and sperm (Li et al., 2003).

4.3 Reproductive performance and mature gonad morphology of triploid and diploid siblings

The reproductive performance and gonad morphology of triploid and diploid sibling *P. monodon* are reported on in detail in Sellars et al., 2011b. Below is a brief summary of the main findings.

4.3.1 Triploid female reproductive performance

Reproductive age triploid female *P. monodon* failed to show any signs of ovarian development and cycling whilst their diploid sibling sisters showed normal ovarian development and cycling. This gross morphological observation was supported by the gonad histology which revealed that triploid female gonads had no typical structure with few developing oocytes and mature ova. Diploid siblings had normal gonad structure with developing oocytes and mature ova. Similar observations to these have been reported for reproductively mature *P. japonicus* and *F. chinesis* triploid females, which are the only other two penaeid prawn species that have had comprehensive gonad assessments of reproductively mature triploids performed

(Sellars et al., 2009; Li et al., 2003). Based on the gross morphological and histological examination of triploid ovaries, results indicate that female *P. monodon* triploids are reproductively sterile.

4.3.2 Triploid male reproductive performance

All spawnings from wild G_0 diploid females were not viable when these females were crossed with triploid males; in stark contrast to all spawnings produced from matings involving G_0 or G_1 diploid males. When the thelyca of the diploid females which were crossed (and so had opportunity to mate) with the triploid males were assessed, no visible spermatophore nor sperm were present indicating that G_1 triploid males may be incapable of impregnating females. It is also possible that the triploid males do mate, but that the spermatophores degrade within the female thelycum.

The lack of viable spawnings from triploid male matings is supported by the gonad histology whereby there was a distinct lack of sperm cells within the primary and secondary lumens of the vas deferens compared to a proliferation of these cell types in diploid siblings. Li et al., 2003 also report a lack of sperm being observed in the vas deferens and spermatophores of reproductively mature triploid *F. chinesis.* Spermatozoa present in the testes of *P. monodon* triploids also lacked the rigid spike required to initiate fertilization, which has also been reported for triploid male *F. chinensis* (Xiang et al., 2006).

5. Conclusion

Based on the findings from this *P. monodon* triploid family, triploid induction will not be a suitable means for skewing sex ratios towards faster growing females for this species. However, triploid female and male *P. monodon* were found incapable of producing viable progeny when crossed with diploids of the opposite sex. Acute reductions in reproductive development of female and male triploid *P. monodon* gonadal tissues were apparent to a point were complete maturation has not occurred. Reproductively mature triploid *P. monodon* were in effect reproductively sterile, indicating that triploidization of this species could provide a means of genetic protection for selectively bred *P. monodon*. Importantly, the practicality and cost-effectiveness of such approaches must be considered in terms of commercial implementation of using triploidy as a means for genetic protection. There are still some significant challenges for applying a shock agent for high levels of triploidy in whole spawnings. The spawning sized pressure chamber tested in this study may provide a means of producing whole triploid spawns in the future, however, significant further effort is still required to optimise this process.

6. References

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Appendix 1: CMAR Scientific Equipment and Technology Prawn Pressure Chamber Design and Specifications Brochure.

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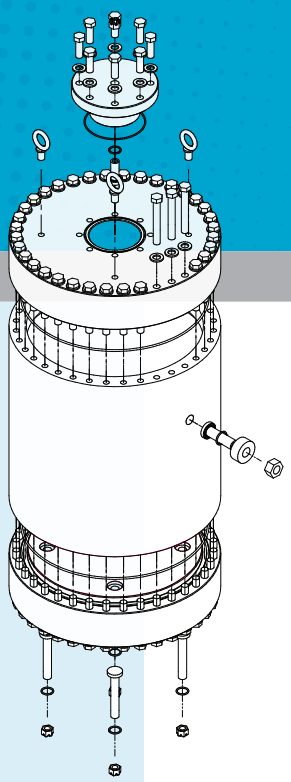
CMAR Scientific Equipment and Technology

Prawn pressure chamber

CSIRO Marine and Atmospheric Research



A pressure chamber to assist in prawn genetics research has been developed by the Scientific Equipment and Technology (SET) group of CSIRO Marine and Atmospheric Research.

The prawn chamber resulted from close collaboration between the SET group and scientists with the CSIRO Food Futures Flagship who are using pressurisation to influence early prawn embryo development.

The chamber needed to be large enough to allow the prawns to swim around and spawn comfortably, and allow for the recirculation of warm sea water in order to provide optimum spawning conditions. The solution was to design a cylindrical pressure vessel (110 litres internal volume) which could withstand an internal water pressure of 3000 psi (equivalent to about 2000 metres below sea level).

The pressure housing was machined from solid hollow round steel to an internal diameter of 550 mm. The end caps were designed to allow for multiple penetrations on the bottom, and a large opening on the top for easy access. The vessel and end caps were hard chromed to provide corrosion resistance.

The water is compressed via two pneumatically driven water pumps operated by a small air compressor built into the frame. Digital and analogue pressure gauges allow for monitoring of system pressure while several electrical and mechanical interlocks prevent over pressurisation of the chamber.

Although the chamber requires manual operation, the system is designed to automatically stop when it reaches a pre-set pressure limit. To add flexibility to the system, a low pressure air bubbling ring was incorporated into the bottom of the chamber, along with a digital I/O port allowing for digital instruments to be run while under pressure and monitored from the outside.

The design of such a large vessel in a tight time frame, combined with a complexity of biological requirements for the prawns to ensure they would spawn, made this project particularly challenging. The SET team was able to pool resources and complete the entire build from design to delivery in only six weeks.

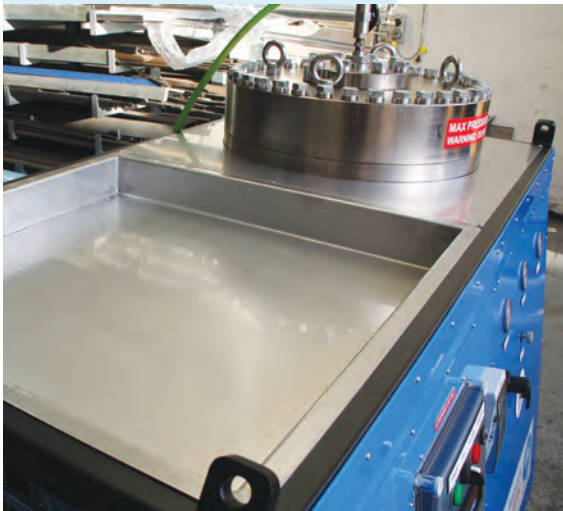
Specifications

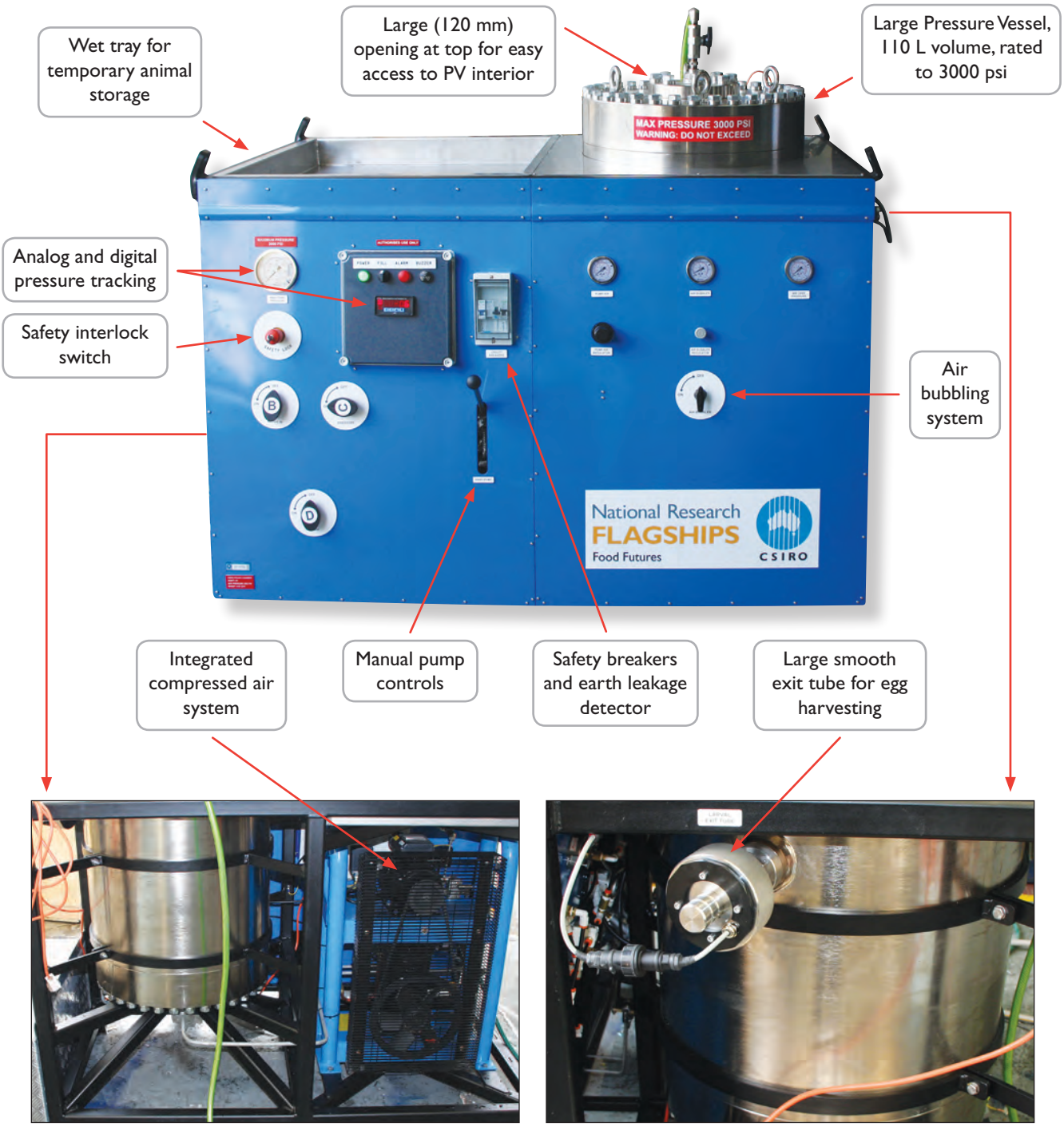
- Tank size (110 litres)
- Pressure rating 3000 psi (equivalent to 2000 m below sea level)
- Vessel size (550 mm internal diameter, 610 mm length)
- Equipment size (1.65 m x 1.05 m x 1.34 m)
- Mass 1200 kg
- AS2971.1H rated

Features

- Large (120 mm) opening at top for easy access to PV interior
- Large smooth exit tube for egg harvesting
- Two water ports, one air port and one electronics port on bottom
- Can accommodate internal sensors via wet-matable connector
- Automated pressure controls
- Built in air bubbling system
- Wet tray work area
- Independent operation from 240VAC
- Mobile platform can be moved between sites
- Upgradable valves for future automation

∧ The completed prawn chamber.





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Australia is founding its future on science and innovation. Its national science agency, CSIRO, is a powerhouse of ideas, technologies and skills for building prosperity, growth, health and sustainability. It serves governments, industries, business and communities across the nation.

Appendix 2: Financial Acquittal Statement.

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

FINANCIAL ACQUITTAL STATEMENT

* To be completed and submitted with your final report

Project No.: 2008/757 **PM:**

Applicant: CSIRO **PI:** Melony Sellars

Funding organisation: Seafood CRC

A. FUNDS RECEIVED **B. PROJECT EXPENDITURE**

MILESTONE NO.	DATE	AMOUNT			
1	1/8/2008	$20,000.00	SALARIES	BUDGET	$110,387.00
2	1/6/2009	$33,000.00		Actual	$112,196.00
3	1/6/2010	$27,000.00	Travel		
4	15/6/2010	$10,000.00		Budget	$15,300.00
5	30/6/2010	$10,000.00		Actual	$7,621.00
6	30/12/2010	$50,000.00	Operating		
7	10/6/2010	$		Budget	$49,313.00
				Actual	$55,183.00
			Capital		
				Budget	$0
				Actual	$0
Total Funds Received	*Total A*	*$150,000.00*	*Total Actual Expenditure*	*Total B*	*$175,000.00*

Amount owed by Seafood CRC/Funding organisation
(Total A – Total B) **$25,000.00**

Comments:

Certified By Financial Officer: _____ **Date:** 30-5-2011

Print name Mark Tuckey

Ph: 07 3833 5975 **Fax:** 07 3833 5505

E-mail: Mark.Tuckey@csiro.au

PM Signature _____ **MD Signature** _____

Date: **Date:**

*☐ FRDC (if applicable)

W:\Finance - Shared Resources\FFF Breed Engineering Theme - R00083\R83-24 - Sellars Seafood CRC Triploid\R83-24 Financial Acquittal_170511.DOC

Triploid Black Tiger shrimp (*Penaeus monodon*) performance from egg to harvest age.

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Keywords: Genetic protection, reproductive sterility, prawn, polyploidy

1.0 Abstract

In this study we induced triploids in a single family of Black Tiger shrimp (*Penaeus monodon*) using chemical shock induction to stop the second meiotic division. The family was reared from egg to harvest age (post-larval stage 184) in a combination of commercial hatchery and experimental controlled environment tank rearing systems. During the rearing phase ploidy, survival, weight, sex ratio and gonad morphology of sibling diploids and induced triploids were periodically assessed. This is the first published report of the culture performance of triploid *P. monodon*. The induction rate of triploids at the nauplii stage was 83.4 ± 0.04% but this proportion had dropped to 62.45% by the time shrimp metamorphosed to the protozeal stage. Proportions of

triploids remained fairly constant throughout the remaining larval and early post-larval stages (ranging from 60.47 to 72.38%), but dropped again to 56.32% by the time shrimp reached harvest age. The reduction in triploid rate from egg to harvest age is thought to be due to a combination of factors including heightened sensitivity of triploids to continual handling and general lower survival of triploids compared to diploids. Once shrimp reached harvest age diploid females were significantly heavier (42.95 ± 2.43 g, P <0.05) than all other categories of shrimp. There was no significant difference in weight (P >0.05) between triploid females, diploid males and triploid males (35.14 ± 2.26 g, 34.02 ± 0.89 g and 31.79 ± 1.56 g respectively). The final sex ratio of harvest age triploid *P. monodon* was 1 female: 1.625 males. Histological examination of harvest age female and male gonads revealed a drastic reduction in the development of both ova and sperm in triploid shrimp compared to diploids. The limited numbers of oocytes and sperm produced in triploids failed to fully mature by harvest age. No sperm were observed within spermatophores of triploids at harvest age when a gross morphological examination was performed and no sperm bundles were seen in the vas deferens. Using the same examination technique, diploid harvest age males had sperm within the spermatophores and vas deferens, and sperm bundles. Results indicate that reproductive sterility is likely for both female and male triploid *P. monodon.*

2.0 Introduction

Selective breeding of the Black Tiger shrimp, *Penaeus monodon*, has become a commercial reality for several international shrimp breeding programs in Australia, India, Malaysia, Vietnam and China. Of these programs, Australia has some of the most advanced selectively bred Black Tiger shrimp lines with demonstrated commercial pond performance sustained across several generations. In the 2009-2010 production season the most advanced of the Australian selected Black Tiger shrimp lines produced on average 17.5 metric tonne/ha which is more than double the harvest yield of unselected lines (Preston et al., 2010). Alongside this impressive improvement in harvest yield, Australia's selected Black Tiger shrimp have improved survival performance, reduced maintenance demands and improved feed conversion efficiency (Glencross et al., 2010).

Currently there are three selectively bred Black Tiger shrimp lines under development in Australia at three collaborating shrimp farms. However, for the rest of the Australian industry to benefit from the national R&D investment in the development of these lines through the purchase of selectively bred post-larvae for grow-out, and for the producers of the selected lines to capitalize on international markets in post-larvae sales, fail-proof genetic protection is currently considered a '*must have*' prior to their sale. As a result, the demand for a fail-proof method of genetic protection in shrimp has never been so great.

To-date there is no commercial method for fail-proof genetic protection of shrimp. Triploid induction is the only methodology that has been trialled experimentally that shows promise of genetic protection however, there are limited reports of triploid induction in Black Tiger shrimp and no studies have reared the larvae through to harvest age and conducted rigorous performance measurements (Sellars et al., 2010). Such triploid performance data is only available for two penaeid shrimp species, *Fenneropenaeus chinensis,* and *Marsupenaeus japonicus* (*F. chinensis:* Li et al., 1999; Li et al., 2002; Li et al., 2003; Xiang et al., 2006. *M. japonicus:* Preston et al., 2004; Sellars et al., 2006; Coman et al., 2008; Sellars et al., 2009). In both species triploid shrimp are reported to be reproductively sterile once they reach sexual maturity.

In this study we induced triploids in a Black Tiger shrimp family using chemical shock induction to stop the second meiotic division. The family was reared from egg to harvest age (post-larval stage (PL) 184, ~6 months old) in a combination of commercial hatchery and experimental controlled environment tank rearing systems. During the rearing phase ploidy, survival, weight, sex ratio and gonad morphology of sibling diploids and induced triploids were periodically assessed.

3.0 Materials and methods

3.1 Spawning and triploid induction

Wild-caught broodstock collected from a population off the coast of Innisfail (17°53'S, 146°01'E), Queensland, Australia were conditioned for spawning at a

commercial hatchery according to standard industry practices. Females that were ripe (ready to spawn) were selected based on gonad development determined by shining a torch beam through the dorsal exoskeleton (Tan-Fermin and Pudadera, 1989). Ripe females were transferred to 100 L flow-through circular spawning tanks filled to 60 L (water flow 0.7 L min^{-1}, water temperature 28.5°C, moderate aeration), and fitted with an automatic spawning detection device (Coman et al., 2003) and an internal 90 μm mesh pen (Fig. 1a).

The spawning detection device detects *P. monodon* embryos whilst still being released from the female. Time zero was taken as the time when the spawning detection device was triggered. For ease of explanation, spawned eggs, whether fertilized or unfertilized, will be referred to as embryos from hereon.

At ~4 min post-spawning the female was removed from the spawning tank and all aeration and the spawning detection device removed. At 6 min post-spawning 8 L of seawater and embryos were gently removed from the spawning tank using a 10 L bucket and set aside (temporarily with no aeration) as a control for estimating hatch rate. At 6 min 40 sec post-spawning 6-dimethylaminopurine (6-DMAP) dissolved in 29°C seawater (made fresh <6 h prior) was added to the remaining 52 L to give a final treatment concentration of 200 μM. Gentle aeration was used immediately after addition of 6-DMAP for ~5 seconds to ensure even mixing.

To achieve a 10 min treatment duration, the mesh pen was slowly lifted from the spawning tank from 16 min post-spawning to 16 min 30 sec post-spawning and subsequently placed in a 1,000 L tank (water flow 1.2 L min^{-1}, water temperature 28.4°C, no aeration) to cease the treatment (Fig. 1b). At 1 h post-spawning the mesh pen was slowly lifted from the 1,000 L tank, embryos gently rinsed to the bottom of the pen (Fig. 1c) and transferred by pouring to a commercial hatching system (Fig. 1d). The 10 L bucket of control embryos was placed in a flow through system to maintain water temperature and given gentle aeration at ~40 min post-spawning.

3.2 Hatch rate calculations, ploidy analysis and commercial hatchery rearing to PL10

At 16 h post-spawning the hatch rate of the control and treatment spawning aliquots were estimated by counting unhatched embryos and nauplii in a 250 mL subsample of the total water volume. Three replicates of 50 nauplii from the bottom chamber of the commercial hatching system (which contains all hatched nauplii with a strong swimming behaviour) and two replicates of 50 nauplii from the controls were sampled and snap-frozen on dry ice for ploidy analysis. Ploidy analysis was performed as described by Sellars et al., 2010 on a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter Australia Pty Ltd). A total of 50,000 shrimp cells were analyzed per sample and the rate of ploidy determined by Quanta Analysis software (Beckman Coulter Australia Pty Ltd).

Once a high triploid induction rate was confirmed by flow cytometry, all nauplii in the bottom chamber of the commercial hatching system were collected and placed in 10 L of 29.8°C seawater. The total number of harvested nauplii in the triploid induced family was estimated from two 250 mL subsamples of the 10 L water volume. Harvested nauplii from the triploid induced family were stocked in a commercial 10,000 L parabolic tank and reared to PL10 according to standard industry practices. As the harvested nauplii from the triploid induced family contained a combination of triploid and diploid individuals, the control nauplii were not reared past nauplius stage as it was not possible to replicate the commercial parabolic tank rearing system accurately with so few shrimp.

During the parabolic tank rearing phase the triploid rate of 35 individual protozoea, 58 individual PL2s and 45 individual PL10s was determined by flow cytometry as described by Sellars et al., 2010 on a total of 10,000 shrimp cells and analyzed as described above. At PL10 shrimp were harvested using standard industry procedures. A random subsample of 200 PL10s were dried for 4 h at 60°C on nylon mesh and placed into a vacuum desiccator for 1 h and weighed in batches of 20 to the nearest 0.0 mg to estimate a mean dry weight per shrimp.

3.3 Rearing from PL 10 to PL184 (harvest age)

The triploid induced family was reared from PL10 to PL184 (harvest age) in controlled environment clear-water tank systems at CSIRO Marine and Atmospheric Research, Queensland, Australia. Shrimp were fed and tanks maintained according to standard CSIRO rearing protocols (Coman, 2002). In brief shrimp were fed a combination of commercial pellets (Lucky Star, Taiwan Hung Kuo Industrial Pty Ltd) and cut fresh squid (*Loligo spp.*) twice daily at approximately 0900 and 1700 hours. Tanks were circular, received flow through seawater (1.6 L min^{-1}) maintained at 28 ± 1°C, had gentle aeration and a lid to reduce light intensity.

At PL10 25 x 100 L bare bottom tanks were each randomly stocked with 150 shrimp and a 1,000 L bare bottom tank was stocked with 5,500 shrimp. Once shrimp reached PL28 they were harvested from these bare bottom tank systems by netting, counted and those originating from 100 L tanks pooled into one 300 L tank and those originating from the 1,000 L tank placed into another 300 L tank. Random samples of 110 individuals from the pool of shrimp stocked in the 100 L tanks and 83 individuals from the (pool of) shrimp stocked in the 1,000 L tank were dried for 4 h at 60°C on nylon mesh, placed into a vacuum desiccator for 1 hr and individually weighed to the nearest 0.0 mg. A random sample from both rearing systems of 74 shrimp was taken and individuals were snap-frozen in liquid nitrogen and ploidy analysis performed as described by Sellars et al., 2010 on a total of 10,000 shrimp cells and analyzed as described above.

At PL28 shrimp were randomly stocked into 2,000 L sand-based tank systems as described by Sellars et al., 2009. In total 5 tanks were each stocked with 200 shrimp originating from the 100 L tanks and 6 tanks were each stocked with 200 shrimp originating from the 1,000 L tank. Once shrimp reached PL60 they were harvested by siphoning out the tank water and physical collection of individuals. The total number of shrimp from each tank was counted and each shrimp weighed to the nearest 0.00 g. One pleopod was non-destructively removed from a random subsample of 110 individuals using sterile forceps and scissors, and haemolymph (20-80 µL) collected from the wound for ploidy analysis as done by Sellars et al., 2009. Haemolymph was immediately added to 150 µL ice-cold anticoagulant solution as described by de la Vega, 2006, ploidy analysis performed as described by Sellars et al., 2010 on a total

of 10,000 shrimp cells and analyzed as described above. The pleopod (which was removed to allow rapid collection of suitable quantities of haemocytes) was discarded.

PL60 shrimp were subsequently pooled and randomly stocked at 99 shrimp across 6 x 5,000 L sand-based tank systems. Once shrimp reached PL184 (harvest age) they were harvested as described for the 2,000 L tanks, the total number of shrimp from each tank counted, and each shrimp was sexed, weighed to the nearest 0.00 g and sampled for individual ploidy analysis as described above for PL60 shrimp.

3.4 Histological gonad and male reproductive tract assessment at PL184

Histological gonad assessment of triploid and diploid siblings from both sexes was performed once shrimp reached PL184. In total12 diploid female, 12 diploid male,12 triploid female and 12 triploid male shrimp were randomly sampled from all 11 tanks (i.e. 20% of the population), cut longitudinally and fixed in Davidson's Fixative for 24 h. Samples were then processed for histological assessment with haematoxylin and eosin stain as described by Bell and Lightner, 1988. General morphology of gonads and surrounding organs were examined using light microscopy and comparisons made between siblings of the different treatment groups. Images were captured using a Zeiss AxioVert microscope, Zeiss AxioCam camera and AxioVision software V4.8 (Carl Zeiss Pty Ltd.).

Male reproductive tract, spermatophore and sperm quality were assessed from triploid and diploid shrimp at PL184. In total 3 diploid and 3 triploid males had their gonads dissected and gross morphology compared by visual examination. Another 2 diploid and 2 triploid males had their spermatophores extracted by electroejaculation. Ejaculated spermatophores were weighed immediately and one of the two spermatophores from each male was randomly selected to estimate sperm quantity. The single spermatophore from each male was placed into a 10 mL tube with 500 µL of 20 µm filtered seawater. Spermatophores were chopped into pieces using fine point dissecting scissors and vortexed for ~1 min. The sperm solution was then diluted to reach approximately 40 sperm cells per 0.04 mm^2 grid square. To accurately determine sperm concentration, and total sperm count, 10 µL of the diluted sperm

suspension was added to the haemocytometer and the normal sperm cells within 0.04 mm^2 grid squares were counted.

3.5 Statistical analyses

The mean weight of PL184 triploid and diploid shrimp was initially analyzed by one-way ANOVA separately for males and females, and later by two-way ANOVA for ploidy rate and sex (SAS Institute Software, 1999). Spermatosomatic index (SSI) of PL184 triploid and diploid males was compared by one-way ANOVA, (SAS Institute Software, 1999).

Where the overall ANOVA for weight and SSI comparisons showed significant differences (P <0.001), the differences between treatments were identified using the least significance difference test (Kotz and Johnson, 1982).

4.0 Results

4.1 Hatching, triploid induction rates and total harvested nauplii from the triploid induced family

The hatch rate of the untreated control was estimated at 78%, which compared to an estimated hatch rate of 65% for the treated shrimp. The two control pooled nauplii samples were determined to be 100% diploid by flow cytometry analysis, whilst the three treatment pooled nauplii samples were determined to be 89.7, 76.7 and 83.8% triploid (average 83.40 ± 0.04%) (Fig. 2). In total 183,000 nauplii were estimated to be harvested from the triploid induced family which were all stocked into the 10,000 L parabolic tank for rearing to PL10.

4.2 Triploidy rate, survival, weight and sex ratio throughout development

The triploidy rate of the triploid induced family from protozoea to PL60 ranged from 60.47 to 72.38% (Fig. 3). An additional observation that resulted from the triploidy analysis of the larval stages was that triploids took longer (approximately 12-24 hours) than diploids to metamorphose through each larval stage. The total triploidy

rate (not separated for sex) from PL60 to PL184 showed a decrease from 72.38 to 56.32% (Fig. 3).

PL184 (harvest age) was the first sample point where shrimp were large enough to be accurately sexed and have their corresponding ploidy rate determined. Of the 261 PL184 shrimp harvested, 21.09 ± 1.87% were triploid female and 35.07 ± 4.62% were triploid male (i.e. ~56.32% triploidy in the harvest population) (Fig. 4). In total there were 56 triploid females, 65 diploid females, 91 triploid males and 49 diploid males (i.e. 1.14 triploid females: 1.33 diploid females: 1.86 triploid males: 1 diploid male).

Survival from PL28-PL60 in both the 100 L tanks and the 1,000 L tank ranged from 65 to 98% but overall was highest in the 1,000 L tank. Overall survival from PL28-PL60 was 70.5% and from PL60-PL184 was 54.0%.

At PL10 the mean dry weight calculated per shrimp was 0.98 ± 0.06 mg, which is estimated to be a mean wet weight per shrimp of 4.91 mg (0.00491 g). At PL28 the mean dry weight of individuals from the 100 L tank rearing systems was calculated per shrimp to be 9.15 ± 1.10 mg, and from the 1,000 L tank rearing system was calculated per shrimp to be 13.17 ± 0.92 mg, which are estimated to be mean wet weights per shrimp of 46.00 mg (0.046 g) and 66.00 mg (0.066 g) respectively. At PL60 the mean wet weight per shrimp was 860.00 ± 0.45 mg (0.86 g).

At PL184, harvest age, diploid females were significantly larger (42.95 ± 2.43 g; P <0.001) than triploid females (35.14 ± 2.26 g; Fig. 5). Diploid males were, however, not significantly larger (34.02 ± 0.89 g; P >0.05) than triploid males (31.79 ± 1.56 g; Fig. 5). There was also no significant difference between the final harvest weight of triploid females and triploid and diploid males (P >0.05; Fig. 5).

4.5 Histological gonad and male reproductive tract assessment at PL184

Atypical ovaries were present in all 12 triploid PL184 females examined histologically (Fig. 6a and b). An acute reduction of developing oocytes was prominent in all triploid females compared to diploid females. Haemal sinuses and associated structural connectives separating each ovarian nodule in triploid females

were incomplete and failed to fully surround each nodule (Fig. 6a). In many nodules the germinal zone of proliferation was not clearly differentiated in triploid females (Fig. 6a). Triploid nodules comprised of a disorganized array of vacuolated oocytes surrounded by loose connective tissue (Fig. 6a). Interestingly many affected nodules contained arrays of presumably follicle cells surrounding the inner nodule periphery (Fig. 6a). In most cases nuclei appeared granular with fragmented and un-condensed chromatin (Fig. 6b). Picknotic nuclei were also observed within affected areas (Fig. 6b). No mature ova were present within any triploid female gonads examined. Very few basophilic staining oocytes were present and were limited to less than two per nodule. In comparison, no gonad abnormalities were observed in the 12 diploid female siblings examined which had normal ovarian development with zones of proliferation and developing oocytes (Fig. 6c).

Gross abnormalities were observed in all 12 triploid PL184 males examined histologically. In general, seminiferous tubules contained undifferentiated spermatocytes (Fig. 7a). Developing spermatogonia were not discernible at tubule periphery and a distinct lack of spermatozoa was noted within the lumens of the vas deferens (Fig. 7b). Those mature sperm present lacked the rigid spike required for the initiation of fertilization as described by (Xiang et al., 2006) in triploid *Fenneropenaeus chinensis*. Seminiferous tubules containing developing spermatogonia were present in very few instances and when observed appeared to be undergoing breakdown and re-absorption, which was also observed in one diploid male. In comparison, no major gonad abnormalities were observed in the 12 diploid male siblings examined with seminiferous tubules containing normal zones of proliferation (Fig. 7c) and mature spermatozoa present within the lumens of the vas deferens (Fig. 7d).

Spermatophores of triploid males were generally less visible during gross morphological observations, their spermatophores being either partially visible or not visible; while spermatophores of diploid males were mostly fully visible. Visual assessment of the dissected male triploid and diploid gonad and spermatophores found no difference in structure. However, no sperm bundles were observed within the vas deferens and spermatophores of triploid males, whereas they were clearly visible within diploid males (Fig. 8). The spermatosomatic index (spermatophore weight

(g)/prawn weight (g) x 100) was significantly greater in diploid males than triploid males (P <0.001) (Fig. 9) and counts of sperm from ejaculated spermatophores found that spermatophores from diploid shrimp contained $8.18x10^6 \pm 1.16x10^6$ sperm cells while those from triploids contained no sperm.

5.0 Discussion

This study investigated the ploidy, survival, weight, sex ratio and gonad morphology over time of sibling *Penaeus monodon* triploids and diploids reared from egg to harvest age (PL184). This is the first comprehensive performance assessment of triploid and diploid sibling *P. monodon*, with only two other penaeid shrimp species being comprehensively studied in comparable experiments in the past, *Fenneropenaeus chinensis* and *Marsupenaeus japonicus* (*F. chinensis:* Li et al., 1999; Li et al., 2002; Li et al., 2003; Xiang et al., 2006. *M. japonicus:* Preston et al., 2004; Sellars et al., 2006; Coman et al., 2008; Sellars et al., 2009). Performance results for this triploid induced *P. monodon* family have some similarities with the triploid induced *F. chinensis* and *M. japonicus* families, however, there are also some marked differences in triploid and diploid sibling performance such as their sex ratios which are discussed.

Triploid rate throughout development, survival and metamorphosis

The rate of triploidy between the nauplius stage (83.4%) and the protozoeal stage (62.4%) dropped by more than 20% in this *P. monodon* family. Similar reductions in the triploidy rate of *M. japonocus* have been observed between these developmental stages for triploidy inductions using the same 6-DMAP shock agent conducted at our research facilities, however, the difference was not typically so great (i.e. a drop in proportions of triploids of 5-10% is common for *M. japonicus*). The higher magnitude of the difference in *P. monodon* could be a result of this species being more fragile compared to *M. japonicus* during these developmental stages. *P. monodon* embryos have been noted in several instances to be significantly more fragile than *M. japonicus* during experimental procedures at our research laboratory (Sellars et al., 2005; Coman and Sellars 2007; Foote et al., 2011) and they are known to be more difficult to rear during the larval stages. Similar to the results for *P. monodon* and *M. japonicus*,

proportions of triploids in *F. chinensis* have been reported to noticeably decrease (by 4 to 43%) in five of eight batches of shrimp between the nauplius and protozoeal stages (Xiang et al., 2006). Results from this *P. monodon* family indicate that triploid nauplii did not survive as well as their diploid siblings.

During the later larval and early post-larval stages, up to PL60, the triploidy rate ranged from 56.32 to 72.38%. Similar observations for *F. chinesis* have been reported whereby early larval survival (i.e. protozoea II to II and Mysis II to postlarval stage) of triploids compared to diploid siblings that are also exposed to the shock agent remains constant (Li et al., 2006). However, in another study on *F. chinensis* by the same research team, which examined triploidy rates (by heat shock induction) in eight batches of shrimp during larval development, four of the batches had a reduction in triploidy of 20% or more between the protozoeal to post-larval stages, whilst three of the batches showed variation in ploidy rate of less than 15% across the larval stage (Xiang et al., 2006), which is similar to what we observed in this study for *P. monodon*. Our results indicate that the triploid *P. monodon* shrimp had similar survival to their diploid siblings throughout the later larval and early post-larval stages, up to PL60.

An additional observation that resulted from the larval ploidy analyses was that triploid *P. monodon* took longer (approximately 12-24 hours) than treated diploids to metamorphose through each larval stage in this chemical induced triploid family. The fact that triploid larvae require longer to metamorphose through the larval stages than treated diploid siblings has also been reported for heat induced triploid *F. chinensis* families with a similar delay time of ~24 hours (Xiang et al., 2006). A possible explanation for this time delay is that triploids have an extra set of chromosomes which may take longer to replicate at every cellular division, slowing development resulting in the time delay observed to metamorphose to the subsequent larval stage.

Once *P. monodon* reached PL184 the triploidy rate dropped to 56.32% (from 72.38% at PL60). This is in contrast to results from *M. japonicus* whereby survival of triploids from PL118 to PL306 was similar to that of diploid siblings (Coman et al., 2008). It is hard to quantify the cause of this drop in triploidy rate in the present study, however; a likely scenario is that the triploid shrimp died more frequently than diploid shrimp as

a result of several factors relating to handling. Firstly, from previous research we know that handling of first generation *P. monodon* once they are past ~40 g is extremely stressful for the shrimp and that they often show lower survival rates after having performance assessment measurements like those done in this study. Secondly, we also know that triploid shrimp do not cope with external sources of stress as well as diploid shrimp, meaning that they are less robust when handled frequently as done in this study (CSIRO unpublished information). Taken together, it is plausible that the observed reduction in triploidy rate between PL60 and PL184 was in part an artefact of the experimental design and data collection process.

To be commercially viable, Australian producers of the selectively bred genotypes currently say that triploidy rates at harvest age would need to be in the range of 80-100% to be viable (Australian Prawn Farmers Association Pers. Com.). To achieve this, further optimization of the induction and rearing procedures would be required, along with assessments under commercial conditions where shrimp are not removed from the rearing system for performance measurements multiple times throughout grow-out. Performing triploid assays on *P. monodon* that are past the first 3-4 years of domestication (i.e. past G_3-G_4) may also result in higher survival and thus triploidy rates; due to the greater robustness of these multiple generation domesticated stocks at all life-history stages (CSIRO unpublished information).

Overall the survival of the triploid family from egg to PL184 was low, however, as the induction was performed on a whole *P. monodon* spawning, suitable numbers remained alive to perform the rigorous performance assessments. The low survival in this study is thought to be due to a combination of unquantified parameters including family performance (noting that this is a single family spawned from a wild-caught female), the initial chemical treatment induction, continual handling throughout the larval and post-larval rearing phases to allow performance assessment to be routinely carried out, and the fact that triploid shrimp are known to be more fragile than diploid shrimp. It is worth noting that, in collaboration with industry partners, our research team helped to develop the Australian industries selected Black Tiger shrimp lines and that during this process we attained similar survivals for first generation (G_1) *P. monodon* progeny reared in the same clear-water tank systems that we are reporting for this triploid G_1 *P. monodon* family. To attain meaningful survival data from a

commercial point of view, survival of triploid *P. monodon* needs to be assessed from egg to harvest age using commercial rearing conditions for all phases of production.

Weight and sex ratio of triploid and diploid siblings throughout development and sex ratios

Weights throughout the performance assessment (i.e. from ages PL10 to PL60 and from PL60 to PL184) were similar to previous studies on G_1 *P. monodon* reared under the same clear-water tank systems with similar densities and diets (CSIRO unpublished information). In this study harvest age, PL184, was the first sample point where shrimp could be accurately weighed, sexed and analyzed for ploidy at the same time. By the time *P. monodon* were PL184 treated triploid females were significantly smaller than the treated diploid females. Similar studies on *M. japonicus* reported that once they reached PL173, treated triploid females are significantly smaller than untreated diploid females but are only generally smaller than treated diploid females (Coman et al., 2008). Although weight data separated by sex is not available for *F. chinensis*, reported triploidy growth rates are slightly different to *P. monodon* and *M. japonicus*, with triploids growing at a comparable rate to diploid siblings during the juvenile stages (Xiang et al., 1998), but having better growth rates by the time they reach 6 months of age, (Xiang et al., 2006) which is an equivalent age to the PL184 *P. monodon* in this study and the PL173 *M. japonicus* in the study by Coman et al., 2008. In contrast to the females, at PL184 *P. monodon* diploid males were not significantly larger than triploid males. In this study there was also no significant difference between the final harvest weight of triploid females and triploid and diploid males. Overall, treated diploid female *P. monodon* grew faster than treated triploid siblings and treated diploid males in this study.

The observed sex ratios of the triploid *P. monodon* in this study are considerably different to those observed for *F. chinensis* and *M. japonicus*. In this study the sex ratio of triploid shrimp was 1 female: 1.625 males. In comparison, triploid *M. japonicus* produced by stopping the same meiotic division using chemical shock are always female (Coman et al., 2008), whilst triploid *F. chinensis* produced by stopping the same meiotic division with heat or chemical shock have a sex ratio of 4 females: 1 male (Li et al., 2003; Xiang et al., 2006). The high proportion of triploid

male *P. monodon* in the present study was not expected and has significant implications for scientific theory on how sex is determined in penaeid shrimp. Based on the results of this triploid family, triploidy in *P. monodon* does not confer predominantly female populations as for *M. japonicus* and *F. chinensis*, indicating that triploidy will not provide a suitable mechanism to skew sex ratios towards the faster growing females.

In summary, the weight of triploid and diploid *P. monodon* needs to be compared when reared in earthen ponds to make meaningful assumptions on their growth performance under commercial culture. Based on the performance of the single family in the present study, results suggest that triploidy will have only a small impact on harvest weights. Of course, if triploidy confers reliable reproductive sterility, this technology could provide sufficient benefits to commercial culture as a mechanism for genetic protection, even without the added advantages of all-female populations.

Gonad morphology

Overall, a drastic reduction in the development of both ova and sperm in triploid shrimp in comparison to diploids was observed. The limited numbers of oocytes and sperm produced failed to show signs of full maturation when examined histologically compared to diploid controls, and both triploid male and triploid female reproductive sterility is probable. Similar results have been reported for *F. chinensis* where triploids have a drastic reduction in both ova and sperm (Li et al., 2003). Despite the present study showing that PL184 triploid *P. monodon* have abnormal gonad tissues, comprehensive reproductive assessments need to be done once shrimp reach reproductive age (i.e. 10-12 months or PL300 to PL360) to provide confirmation.

Using gross morphological assessment techniques there were no sperm or sperm bundles observed within the vas deferens and spermatophores of male triploid shrimp at age PL184. This is in contrast to the observations of Li et al. 2003 and Xiang et al. 2006 who observed small numbers of sperm cells within the vas deferens and spermatophore of some triploid male *F. chinensis*. However, in synergy with the histological assessments performed in the present study, they also found that all sperm cells observed from triploid *F. chinensis* males were abnormal. The absence of sperm

cells within the spermatophores of triploid shrimp at this age using the gross morphological assessment technique indicates that no or few sperm cells are being generated or they are unable to travel from the testes to the spermatophore. These results indicate that at age PL184 triploid shrimp are reproductively delayed or impaired, suggesting potential reproductive sterility. However, further assessments to confirm this are required once shrimp reach reproductive age (PL300-360).

6.0 Conclusion

Based on the findings from this *P. monodon* triploid family, triploid induction will not be a suitable means for skewing sex ratios towards faster growing females for this species. However, triploid induction could potentially provide a method of reproductive sterilization with harvest age gonad assessments demonstrating a lack of reproductive capacity compared to diploids. Comprehensive reproductive performance assessments of the triploid females and males compared to their diploid siblings need to be completed once they reach reproductive (broodstock) age (PL300-PL360; 10-12 months) to conclusively confirm reproductive sterility. When trialled in later generation domesticated *P. monodon* i.e. G_3, G_4 or beyond, it is plausible that results may be more favourable in terms of robustness of larvae to the triploidy induction process; and that benefits of sterility alone would likely be sufficient for the faster growing multiple generation selected stocks.

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Figure captions

Figure 1. A) A 100 L spawning tank fitted with an automatic spawning detection device and an internal 90 μm mesh pen, B) The 90 μm mesh pen placed in a 1,000 L tank with seawater to cease 6-DMAP treatment, C) Embryos being gently rinsed to the bottom of the pen at 1 h post-spawning prior to transfer into D) the commercial hatching system.

Figure 2. Flow cytometry outputs from the three treatment pooled nauplii samples A) 89.7% triploid, B) 76.7% triploid and B) 83.8% triploid (average 83.40 ± 0.04%). The pink peak corresponds to the chicken red blood cell control, blue refers to the diploid nauplii cells and red refers to the triploid nauplii cells.

Figure 3. Triploidy rate of the triploid induced *Penaeus monodon* family throughout development and the number (n) of pools or individuals analyzed. Samples were pooled for analysis of nauplii only and therefore S.E bars are only presented for this life-history stage. Ploidy of all other life-history stages was measured on individual shrimp. Triploidy data presented above was not separated for males and females at any of these life-history stages.

Figure 4. Triploidy rate and sex of PL184, harvest age *Penaeus monodon* ± S.E.. Percentages are calculated for the total population harvested, n=261. Standard errors are calculated based on the numbers of each category per tank.

Figure 5. Mean weight of PL184, harvest age, triploid and diploid females and males ± S.E. from the triploid *Penaeus monodon* family. ANOVA and a least significance difference test of female weight data showed a significant difference between the ploidy rates as shown by uppercase letters at $P < 0.001$. ANOVA of male weight data showed no significant difference between the different ploidy rates (n.s.d) at $P < 0.05$.

Figure 6. Haemotoxylin and eosin stained cross sections of A) a triploid PL184 female gonad (10X magnification) showing a complete reduction of developing oocytes, incomplete haemal sinuses and associated structural connectives (open arrows), and presumptive follicle cells (shaded arrows) surrounding the inner nodule

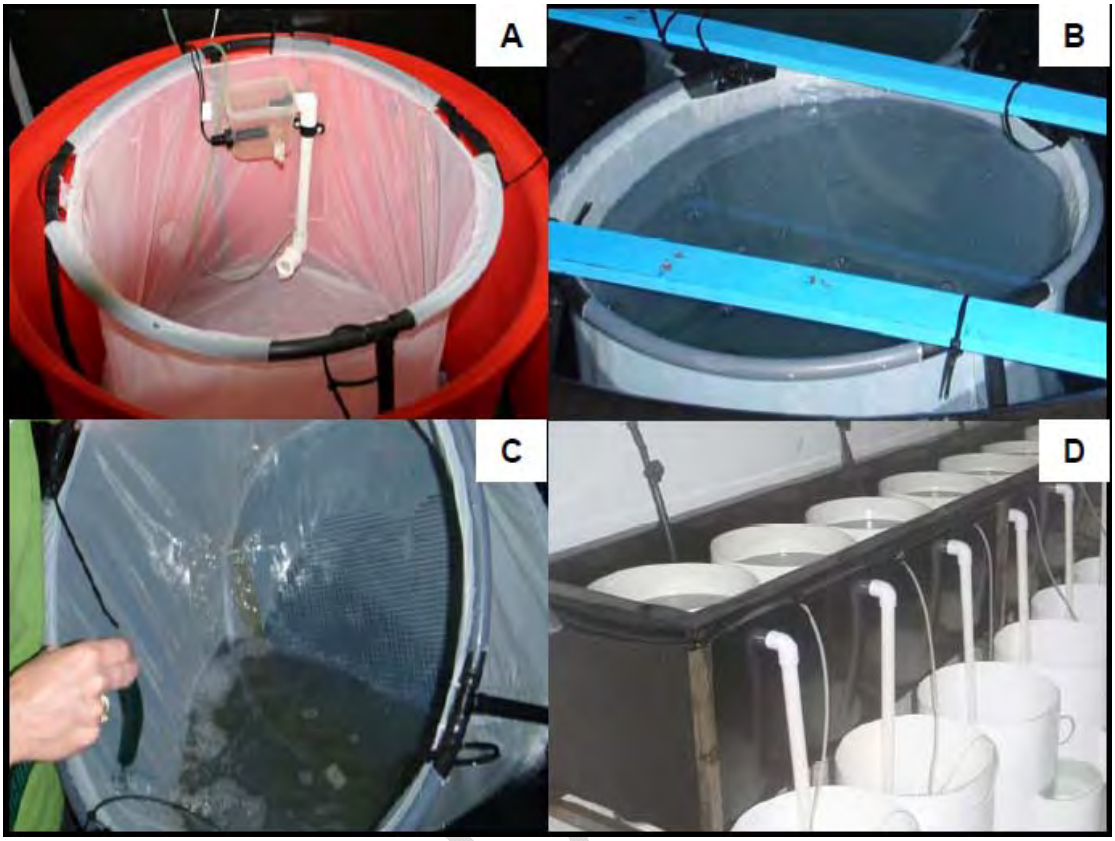
periphery; B) a triploid PL184 female gonad (20X magnification) showing granular nuclei with fragmented and un-condensed chromatin, and picknotic nuclei (open arrows), and; C) a diploid sibling PL184 female gonad (10X magnification) showing normal ovarian development with a zone of proliferation (open arrows) and developing oocytes (shaded arrows).

Figure 7. Haemotoxylin and eosin stained cross sections of A) triploid PL184 male seminiferous tubules (10X magnification) with undifferentiated spermatocytes (open arrow); B) triploid PL184 male vas deferens (10X magnification) with only a few spermatozoa (open arrows); C) sibling diploid PL184 male seminiferous tubules (20X magnification) with normal differentiated spermatocytes (spermatogonia, open arrow, and mature spermatozoa, shaded arrow), and; D) sibling diploid PL184 male vas deferens (20X magnification) with mature spermatozoa (open arrows).

Figure 8. Reproductive tract from A) a PL184 diploid male and B) a PL184 triploid male sibling showing a lack of sperm bundles in triploids. VD = vas deferens, TA = terminal ampule, T = testes, SB = sperm bundles.

Figure 9. Spermatosomatic index (SSI) of triploid and diploid PL184 males, calculated by determining the percentage of ejaculated spermatophore weight to total prawn weight. Bars with different superscripts are significantly different (P <0.001).

Figures



685 **Figure 1**

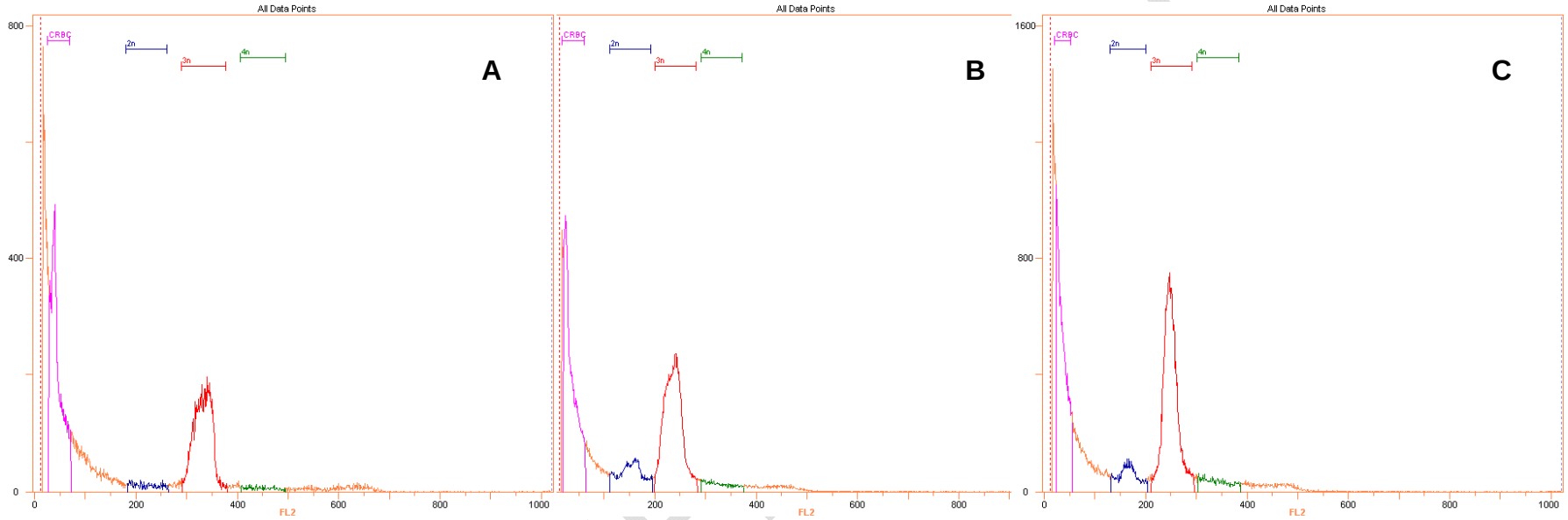
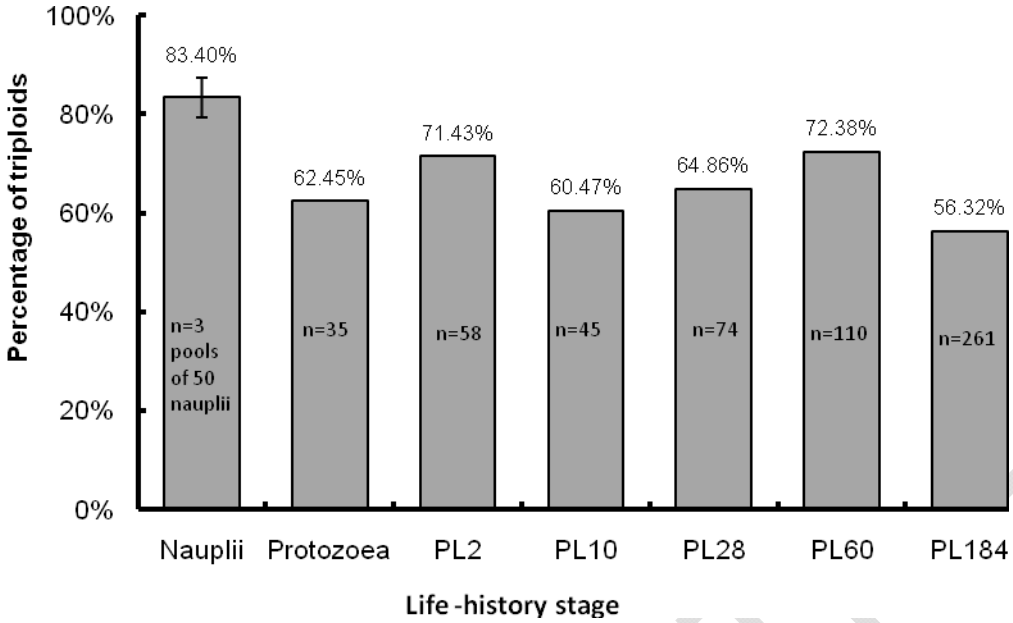


Figure 2



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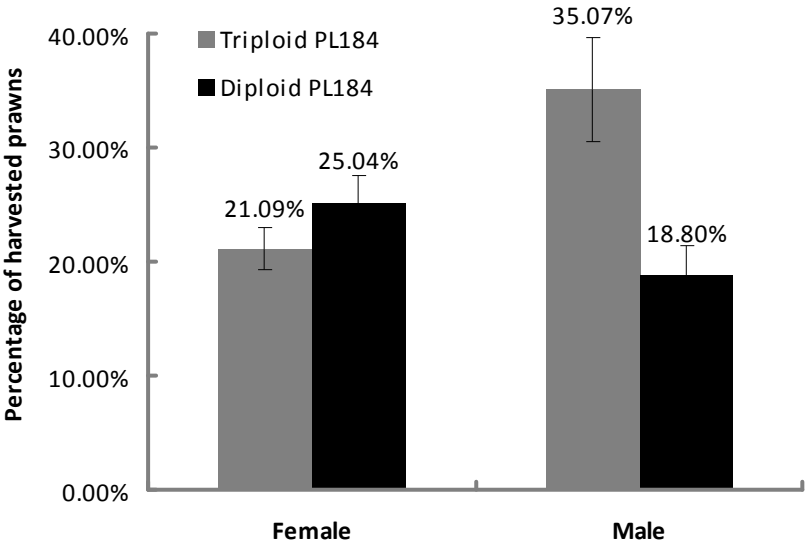
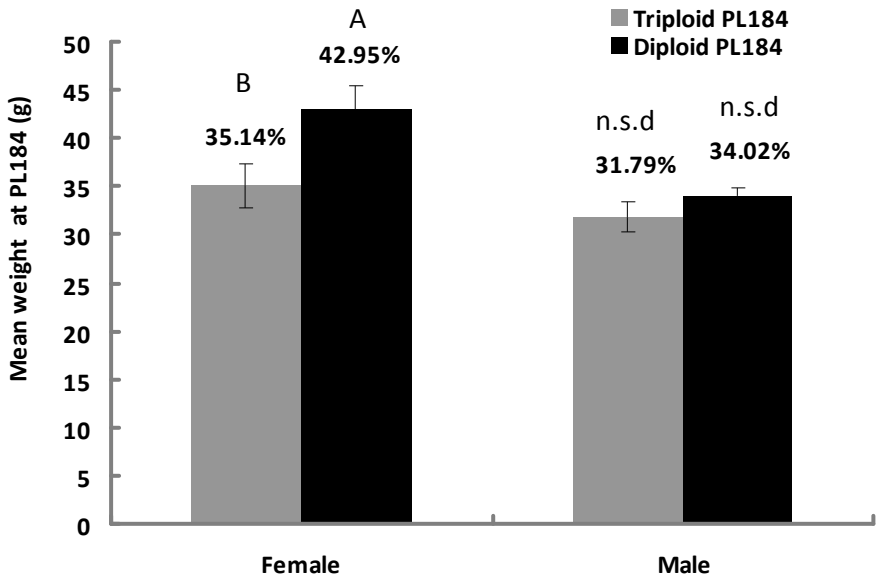


Figure 4



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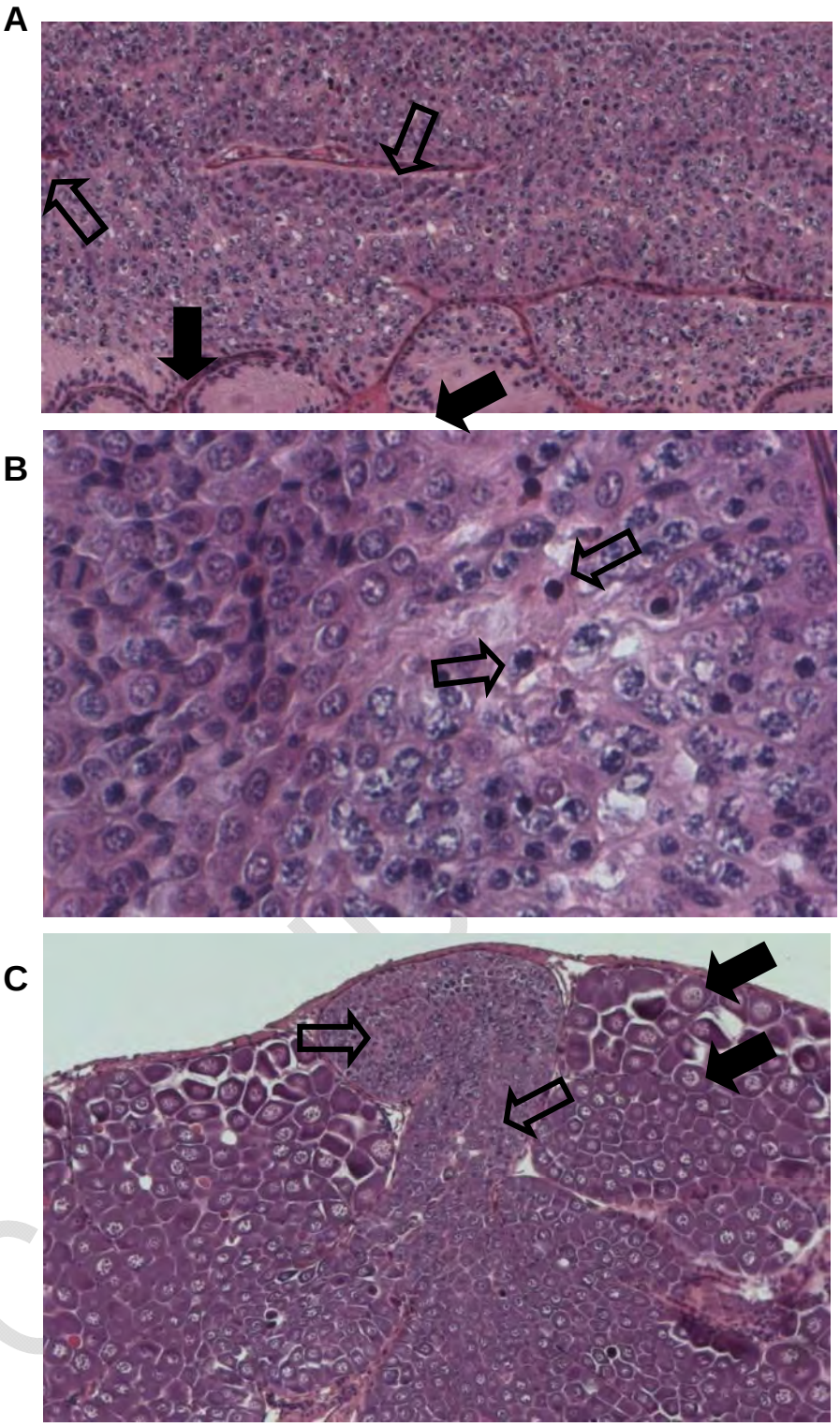

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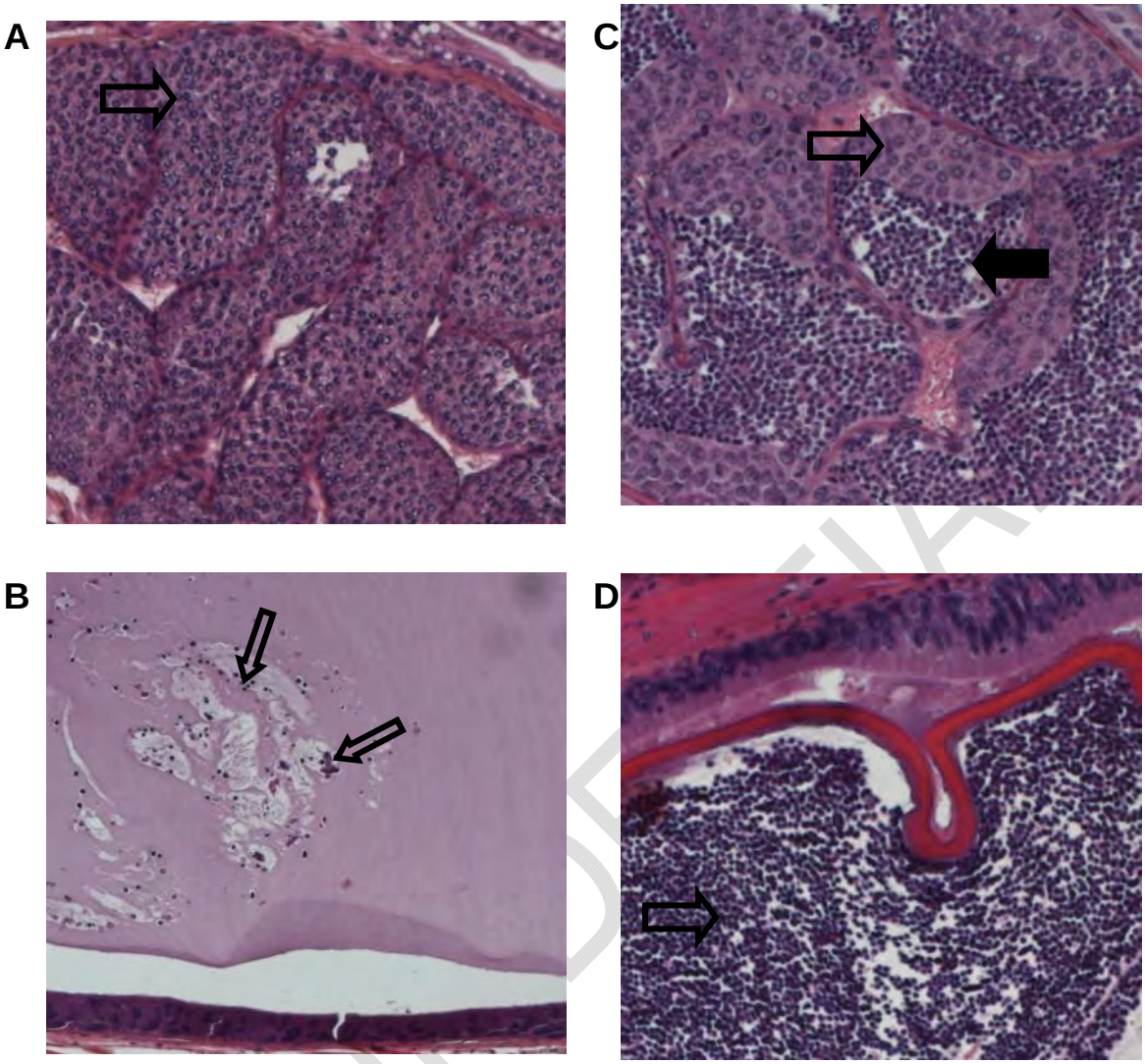

Figure 7

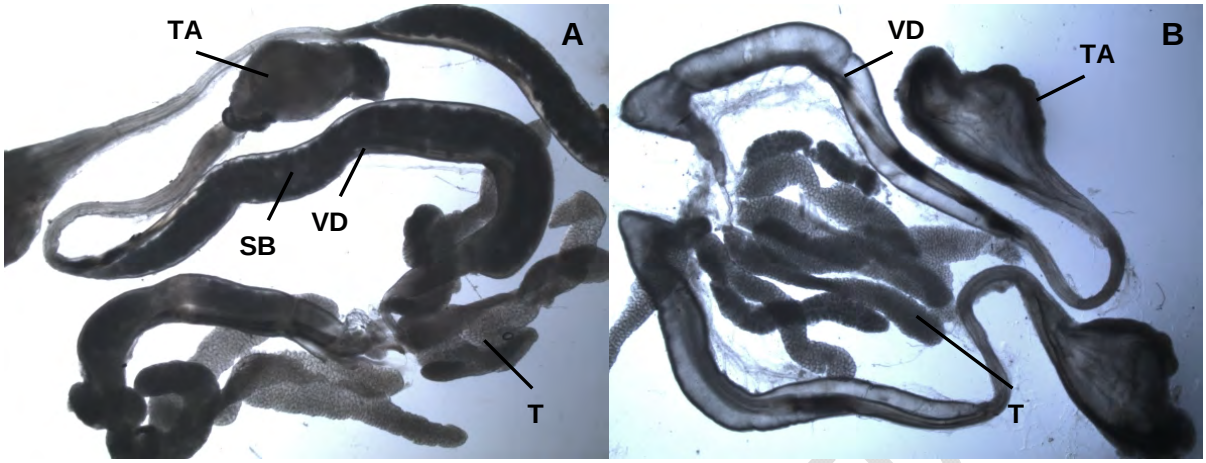

Figure 8

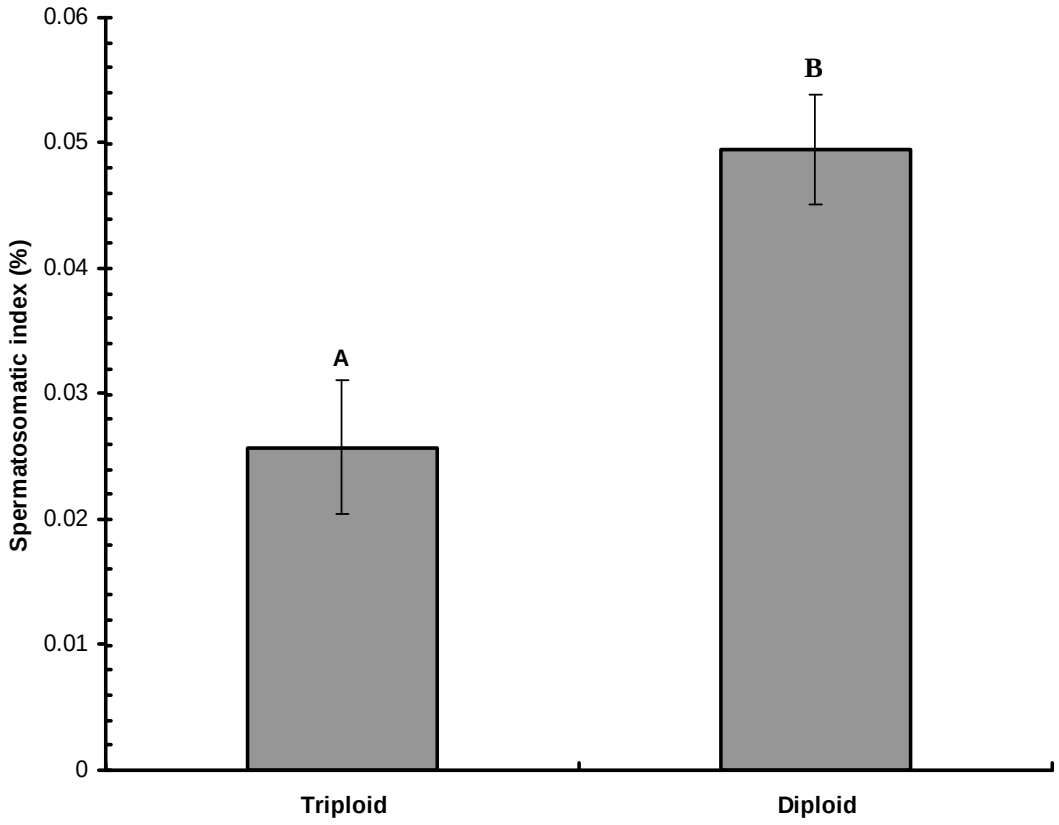

Figure 9.

Reproductive performance and mature gonad morphology of triploid and diploid Black Tiger shrimp (*Penaeus monodon*) siblings.

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1.0 Abstract

In this study we took a harvest age Black Tiger shrimp (*Penaeus monodon*) family with sibling triploids and diploids of both sexes (approx. 6 months old) and reared them to reproductive maturity (approx. 10.5 months old). Triploid and diploid siblings were then crossed with wild caught females and males, conditioned for spawning for 55 days, and a comprehensive 16 day reproductive performance trial was undertaken. During the trial, ovarian development, spawning frequency, fecundity and hatch rate were assessed for the different crosses. At the end of the trial, gonad morphology, male reproductive tract assessments and thelycum impregnation rates of the wild female x triploid male cross were determined. After ablation, ovarian development and ovarian cycling (stage 0 to stage 4) between wild G_0 diploid and G_1 diploid females was not significantly different, whilst G_1 triploid females failed to show any signs of ovarian development and cycling, remaining at ovary stage 0 throughout the trial and thus resulting in no G_1 triploid female spawnings. When assessing first spawnings only, there were 10 G_0 diploid female x G_0 diploid male spawnings and 9 G_0 diploid female x G_1 diploid male spawnings, all of which produced viable nauplii. In comparison, there were 7 G_0 diploid female x G_1 triploid male spawnings, none of which produced viable nauplii. The histological assessments of female and male gonads and male reproductive tract assessments showed impaired reproductive development in both triploid female and triploid male gonadal tissues (compared to sibling diploids and wild shrimp) to a point were complete maturation had not occurred. Diploid siblings and wild comparisons showed normal gonad tissue in all female and males examined. The thelycum of 16 wild G_0 diploid females that were crossed with G_1 triploid males had no visible spermatophore (and so no sperm) present when destructively examined, suggesting that G_1 triploid males are incapable of developing viable spermatophores and mating with females. This study demonstrates that the triploid *P. monodon* females and males are incapable of producing viable gametes and are thus reproductively sterile.

2.0 Introduction

Recent success in the development of high-performance selectively bred Black Tiger shrimp lines and an expanding shrimp grow-out industry seeking access to these lines is driving the demand for selectively bred post-larvae for commercial grow-out around the world. As these stocks are owned exclusively by breeding programs or companies who typically want to retain their genetic investment in development of the lines, access to post-larvae by other grower companies can be restrictive. Many of the broodstock companies producing these selected lines consider fail-proof genetic protection a '*must have*' prior to the widespread sale of selectively bred post-larvae. Consequently, development of a technique to confer reproductive sterilization and thus fail-proof genetic protection for the Black Tiger shrimp, *Penaeus monodon,* is one of the top R&D priorities for the global *P. monodon* shrimp farming industry.

Triploid induction is currently the only known method to confer reproductive sterility and thus genetic protection in penaeid shrimp species. Despite being experimentally studied in most commercially important shrimp species around the world, comprehensive studies on the survival, growth and sex ratio of triploid and diploid siblings from egg to adult has only been reported for three penaeid shrimp species, *Fenneropenaeus chinensis, Marsupenaeus japonicus* and *P. monodon* (*F. chinensis:* Li et al., 1999; Li et al., 2002; Li et al., 2003; Xiang et al., 2006. *M. japonicus:* Preston et al., 2004; Sellars et al., 2006; Coman et al., 2008; Sellars et al., 2009. *P. monodon*: Sellars et al., 2011 in press). Of these, evaluations of reproductive performance and mature gonad morphology of triploids compared to diploid siblings at broodstock-age has only been studied in *F. chinensis* and *M. japonicus* (Sellars et al., 2010).

Triploid *F. chinensis* and *M. japonicus* females of broodstock-age are both reported to have reduced gonad tissue which is not capable of producing viable gametes compared to their diploid siblings who have normal gonads and produce viable gametes. (*F. chinensis*: Li et al., 2003; Xiang et al., 2006. *M. japonicus*: Preston et al., 2004, Sellars and Preston, 2008). Triploid *F. chinensis* males of broodstock-age are thought to be incapable of fertilization as they have abnormal sperm morphology and no spike on the sperm (Li et al., 2003; Xiang et al., 2006; Xie et al., 2008).

In this study, we take a harvest age *P. monodon* family with sibling triploids and diploids of both sexes (approx. 6 months old) and rear them to reproductive maturity (approx.10.5 months old). Triploid and diploid siblings were then crossed with wild caught males and females, conditioned for spawning and a comprehensive reproductive performance trial was undertaken. During the trial, ovarian development, spawning frequency, fecundity and hatch rate were assessed for the different treatment groups. At the end of the trial, gonad morphology, male reproductive tract assessments and thelycum impregnation rates of the wild female x triploid male cross were determined.

3.0 Materials and methods

3.1 Source and rearing of stocks

In a previous study, a first generation (G_1) *Penaeus monodon* triploid induced family was produced at a commercial hatchery using 6-dimethylaminopurine to stop the second meiotic division (Sellars et al., 2011 in press). The family was reared to PL_{10} under standard commercial conditions and from PL_{10} to PL_{184} (~6 months old) in controlled environment tank systems as described by Sellars et al., 2011 in press. This study took 20 G_1 individuals from each of the four treatment classes; triploid females,

diploid females, triploid males and diploid males (80 shrimp in total), and reared them to reproductive maturity.

At 6 months of age all 80 individual G_1 sibling shrimp had their ploidy level non-destructively determined by flow cytometry as described by Sellars et al., 2010 for shrimp haemocytes. All individuals were eye-tagged with unique identifiers, weighed, and females randomly stocked across 2 x 10,000 L tanks and males randomly stocked across 2 x 5,000 L tanks (20 shrimp per tank). Tanks had a sand-substrate and sub-sand filtration system as described by Sellars et al., 2009, received 3.5 L min^{-1} filtered (60 µM) 28 ± 1°C seawater, had a10 mm thin walled polycarbonate (Polygal Inc.) lid to reduce light intensity and were exposed to a 12 h light: 12 h dark photoperiod. Shrimp were fed *ad libitum* twice daily at 0900 and 1700 hours on a combination of commercial pellets (Lucky Star, Taiwan Hung Kuo Industrial Pty Ltd), squid (*Nototodarus sp.*) and green-lipped mussels (*Perna sp.*). Once G_1 shrimp reached PL_{315} (~10.5 months old) they were harvested from their tanks, counted and stocked into one of four 10,000 L tanks (as described above) according to their experimental cross as described below.

To complete the experimental crosses, a total of 58 female and 58 male wild (G_0) *P. monodon* broodstock were collected from a population off the coast of Innisfail (17°53'S, 146°01'E), Queensland, Australia. These G_0 females and males were eye-tagged with unique identifiers and placed into the same four 10,000 L tanks according to their experimental cross as described below.

3.2 Experimental crosses and maturation conditioning

To undertake a rigorous reproductive performance assessment of the G_1 triploid and diploid, female and male siblings, a combination of G_0 wild crosses were performed. In two separate tanks, G_0 diploid females were crossed with G_1 diploid males and G_1 triploid males (Table 1; Tank 1 and 2 respectively). In another two tanks, a combination of G_1 diploid females, G_1 triploid females and G_0 diploid females were crossed with G_0 diploid males (Table 1; Tank 3 and 4). In total there were five cross combinations with 16 to 22 females in each.

Following the stocking of shrimp into their experimental crosses, a 55 day maturation conditioning was undertaken. During this time shrimp were not handled and tank disturbances were kept to a minimum. Shrimp were fed *ad libitum* twice daily at 0900 and 1700 hours with a diet consisting of (% dry weight) 10% commercial pellets (Lucky Star, Taiwan Hung Kuo Industrial Pty Ltd), 55% squid (*Nototodarus sp.*), 20% green-lipped mussels (*Perna sp.*), 5% ox liver and 10% polychaetes (*Diopatra sp.*). Tanks were maintained the same as described above except they received a 10 h light: 14 h dark photoperiod.

3.3 Reproductive performance trial

After the 55 day maturation conditioning period, once G_1 shrimp had reached PL_{370} (approx. 12.5 months of age), the number of shrimp in each tank were counted, all females were unilaterally eye-stalk ablated using hot forceps and their subsequent reproductive performance assessed over a 16 day period.

Females were examined daily for ovarian maturation. Ripe females (Tan-Fermin and Pudadera, 1989) were transferred to circular spawning tanks (0.66 m dia., water flow 0.7 L min^{-1}, water temperature 29°C, fitted with a 60 µM outlet screen) filled to 80 L and allowed to spawn. Spawning tanks were checked for spawns every 30 min during

the spawning window (approx. 4 h) and assessment of fertilization rates of the eggs after the first, second or third mitotic division was determined on a subset of approx. 200 individual embryos as done by Coman et al., 2007.

After spawning, or if the ovaries had regressed, females were weighed and returned to their maturation tank. For all spawnings, eggs per spawning were estimated from the total number of eggs collected in three 250 mL samples taken from the spawning tank water between 4 h and 8 h post-spawning. Eggs were then allowed to hatch in the spawning tanks and nauplii numbers per spawning were estimated from the total number of nauplii collected from three 250 mL samples taken 2 to 5 h after observation of first hatching (after thorough mixing to ensure eggs and nauplii were homogeneous within the water).

Histological gonad morphology of G_1 triploid and G_1 diploid siblings, and of G_0 diploid shrimp from both sexes was assessed at the end of the reproductive performance trial. In total three shrimp from each of the treatment categories (G_1 triploid female, G_1 diploid female, G_1 triploid male, G_1 diploid male, G_0 diploid female and G_0 diploid male) were sampled, having their gonads dissected out and fixed in Davidson's fixative for 24 h. Fixed samples were then processed for histological assessment with haematoxylin and eosin stain as described by Bell and Lightner, 1988. General morphology of gonads was examined using light microscopy and comparisons made between the different treatment groups. Images were captured using a Zeiss AxioVert microscope, Zeiss AxioCam camera and AxioVision software V4.8 (Carl Zeiss Pty Ltd.).

G_1 triploid and diploid, and G_0 diploid male reproductive tract, spermatophore morphology and sperm quantity were also assessed at the end of the reproductive performance trial. In total, three males from each of the three treatment categories (G_1 triploid male, G_1 diploid male and G_0 diploid male) had a single spermatophore extracted by dissection. The single spermatophore from each male was placed into a 10 mL tube with 500 µL of 20 µm filtered seawater. Spermatophores were chopped into pieces using fine point dissecting scissors and vortexed for ~1 min. A 10 µL aliquot of the sperm suspension was placed onto a microscope slide and observed under light microscopy at 40 x magnification. Observations were made on presence and morphology of sperm.

At the end of the reproductive performance trial, the thelycum of 16 G_0 diploid females that were crossed with G_1 triploid males was cut from the female and visually examined for the presence of spermatophore(s). The thelycum was gently scraped with a scalpel blade and washed with 500 µL sterile shrimp saline solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA.Na2, 10 mM HEPES, pH to 7.3) which was collected and examined under light microscopy for the presence and morphology of sperm.

3.4 Measures of reproductive performance and statistical analyses

Reproductive performance was expressed in terms of the female weight at first spawning, eggs per spawning, nauplii per spawning, hatch rate per spawning, percentage of spawnings that hatched and percentage of developing embryos per spawn. Performance measures were analyzed by ANOVA (SAS Institute Software, 1999). Where the overall ANOVA for performance measure comparisons showed significant differences (P <0.001), the differences between treatments were identified using the least significance difference test (Kotz and Johnson, 1982).

4.0 Results

4.1 Ovarian development, cycling and spawning frequency

After ablation, ovarian development and ovarian cycling (stage 0 to stage 4) between G_0 diploid and G_1 diploid females was not significantly different, whilst G_1 triploid females failed to show any signs of ovarian development and cycling, remaining at ovary stage 0 throughout the trial. As a result there were no G_1 triploid female spawnings. When assessing first spawnings only, there were 10 G_0 diploid female x G_0 male diploid spawnings and 9 G_0 diploid female x G_1 diploid male spawnings, all of which produced viable nauplii. In comparison there were 7 G_0 diploid female x G_1 triploid male spawnings, none of which produced viable nauplii. Despite the ovarian cycling in the G_1 diploids being comparable to that of their wild counterparts, only a single spawning was achieved in the crossing with G_0 males, and this was not fertile.

4.2 Reproductive performance of females and males

There were no significant differences (P >0.05) between the weights of females in the different treatment categories at time of ablation, and no difference in the weight of those which spawned (Table 2). All crosses with diploid females, whether G_0 or G_1 in origin, produced spawnings and thus eggs. Of those crosses which had more than one spawning (i.e. excluding the G_1 diploid x G_0 diploid cross which only had one spawn), nauplii were only produced when the diploid female was crossed with a diploid male, regardless of whether G_0 or G_1 in origin (Table 2). No nauplii were produced from diploid female spawnings when crossed with G_1 triploid males. As mentioned above, there were no G_1 triploid female spawnings as triploid females did not produce mature gonads. Overall the performance of the G_0 diploid x G_1 diploid cross and the G_0 diploid x G_0 diploid cross was similar, whilst the G_0 diploid x G_1 triploid cross and the G_1 triploid x G_0 diploid cross did not produce any viable progeny.

When comparing the effects of the different male treatments (G_0 diploid, G_1 diploid or G_1 triploid) on G_0 female spawnings, there was no significant difference (P >0.05) between the fecundity, percentage of spawnings that hatched and hatch rates of G_0 diploid male and G_1 diploid male spawns. The percentage of spawnings that were fertilized that hatched and hatch rates were however significantly lower (P <0.05) for G_1 triploid male spawnings when compared to G_0 and G_1 diploid male spawnings.

4.3 Histological gonad morphology

Histological assessment of the three G_1 triploid female gonads revealed that the number of developing oocytes was severely impeded. Few developing basophilic-staining pre-vitellogenic oocytes and eosinophilic-staining vitellogenic mature ova were observed within ovarian nodules (Fig 1a). The connectives surrounding each ovarian lobe of the triploids appeared normal, however the structure of ovarian nodules contained within were grossly atypical (Fig. 1b). Nodule connective tissues, follicle cells and haemal sinuses partitioning individual nodules in many cases were absent and the germinal layer or zone of proliferation was not distinguishable. Affected nodules comprised of highly vacuolated cells, presumed to be oogonia, surrounded by loose connective tissue. Oocyte nuclei were pycnotic in some cases, and nucleoli appeared disrupted with varying degrees of fragmented chromatin. Limited numbers of follicle cells were present in remnant nodule connectives at the lobe periphery.

G_1 triploid females had few discernable nodules with typical structure. Nodule periphery connectives, follicle cells, germinal layer and haemal sinuses appeared normal; however the nodules were void of oogonia and contained only loose connective tissue. A small number (less than five) of basophilic-staining oogonia were observed in less than ten nodules. Oogonia appeared abnormal with enlarged nuclei containing fragmented chromatin and cells were boarded by loose connective tissue. Haemal sinuses were present but only surrounded limited numbers of oocytes compared to large numbers of developing oocytes and mature ova in diploid sibling controls (Fig. 1c). The ovaries of one G_1 triploid female presented palely-staining eosinophilic ova within structurally-impaired nodules located adjacent to acute nodules devoid of developing oocytes, however no peripheral rod-like bodies were observed within the cytoplasm of the mature ova present.

In the age-matched sibling comparison, the three G_1 diploid females, and case control comparison, the three G_0 diploid females, normal ovaries with prolific numbers of developing oocytes and mature ova within the ovarian nodules were observed along with normal cellular organization (Fig. 1d). Overall, the development of the ovary was grossly impeded in all triploid G_1 females examined whilst G_1 and G_0 diploid females had normal ovary morphology. The lack of developing oocytes and absence of rod like bodies in mature ova of triploids indicates normal ovarian maturation has been interrupted. Full maturity in G_1 triploid females may never be achieved and the histology indicates that these females are reproductively sterile.

Histological assessment of the three G_1 triploid males revealed abnormalities in the gonads of all examined shrimp. A distinct lack of sperm cells within primary and secondary lumens of the vas deferens was observed in all cases (Fig. 2a). Spermatozoa present in the testes lacked the rigid spike required to initiate fertilization as described in triploid *Penaeus chinensis* by (Xiang et al., 2006). Atypical seminiferous tubules were observed in all triploid G_1 males (Fig. 2b). Tubules contained undifferentiated stroma, presumably spermatocytes, surrounded by loose connective tissue. Cells were vacuolated in many cases and nucleoli were granular and uncondensed in appearance. Tubules were void of spermatagonia, nurse cells and developing spermatocytes. No structural abnormalities were observed, connectives of both the outer testicular lobe and those associated with tubule haemal sinuses appeared normal. The lack of developing spermatagonia, mature spermatozoa and the absence of rigid spikes indicate complete sperm maturation has not occurred and indicates that the G_1 triploid males will be reproductively sterile. In the age matched sibling comparison, the three G_1 diploid males, and case control comparison, the three G_0 diploid males, normal gonads with prolific numbers sperm cells within the primary and secondary lumens of the vas deferens (Fig. 2c) and normal seminiferous tubules with cellular differentiation (Fig. 2d) were observed.

4.4. Male reproductive tract assessment and wild female x triploid male thelycum assessment

Spermatophores of G_1 triploid males were only partially visible during external observations, while spermatophores of G_0 and G_1 diploid males were mostly fully visible. Visual assessment of the dissected male G_1 triploid, and G_0 and G_1 diploid gonad and spermatophores found no difference in structure. However, no sperm bundles were observed within the vas deferens and spermatophores of triploid males, whereas they were clearly visible within diploid males (Fig. 3). Spermatophores of G_0 and G_1 diploid males contained abundant sperm with normal morphology while those from G_1 triploid males contained no sperm. The thelycum of 16 wild G_0 diploid

females that were crossed with G_1 triploid males had no visible spermatophore nor sperm present when destructively examined.

5.0 Discussion

This study took a harvest age *P. monodon* family with sibling triploids and diploids of both sexes and reared them to reproductive maturity. Triploid and diploid siblings were then crossed with wild caught males and females, conditioned for spawning and a comprehensive reproductive performance trial was undertaken. Observations on ovarian development, spawning frequency, fecundity, hatch rate, gonad morphology and thelycum impregnation rates conclusively demonstrate that the triploid female and male *P. monodon* were incapable of producing viable progeny in this study.

5.1 Triploid female reproductive performance

Reproductive age triploid female *P. monodon* failed to show any signs of ovarian development and cycling in the present study whilst their diploid sibling sisters showed normal ovarian development and cycling. This gross morphological observation was supported by the gonad histology which revealed that triploid female gonads had no typical structure with few developing oocytes and mature ova. Diploid siblings had normal gonad structure with developing oocytes and mature ova. Similar observations to these have been reported for reproductively mature *P. japonicus* and *F. chinesis* triploid females, which are the only other two penaeid shrimp species that have had comprehensive gonad assessments of reproductively mature triploids performed. Sellars et al., 2009 reported that triploid female *P. japonicus* have no observable stage 3 or 4 ovary tissue at PL_{360} whilst sibling diploid females showed normal stage 3 and 4 ovarian development. Similarly, Li et al., 2003 also reported a lack of gonad development in triploid female *F. chinesis* compared to diploid siblings, whilst also making similar histological observations to those reported here whereby triploid ovaries were much smaller with very few developing oocytes and mature ova, and no typical gonad structure. Based on the gross morphological and histological examination of triploid ovaries, results indicate that female *P. monodon* triploids are reproductively sterile. It is worth noting that the histological observations of the triploid and diploid female *P. monodon* gonads at reproductive age (approx. 12.5 months) in this study are similar to those reported for this same family when they were at harvest age (approx. 6 months) (Sellars et al., 2011 in press).

It should also be noted that, despite the comparable ovarian cycling in the G_1 diploid females compared to the wild G_0 diploid females and normal gonad morphology, only a single G_1 diploid female x G_0 diploid male spawning was achieved, and this was not fertile. This low spawning performance is not an 'atypical' result of an early generation domesticated *P. monodon* female and has been observed previously throughout the process of domestication of several *P. monodon* lines (CSIRO Unpublished Information). The absence of fertilization in the one spawning that did occur is likely due to egg quality. Certainly, the consistent performance of the G_1 diploid males, compared to G_0 diploid males, suggests that this effect of early generation rearing seems less significant for males than females, and has been reported previously by our research team (Coman et. al., 2006).

5.2 Triploid male reproductive performance

All spawnings from wild G_0 diploid females were not viable when these females were crossed with triploid males; in stark contrast to all spawnings produced from matings involving G_0 or G_1 diploid males. When the thelycums of the diploid females which

were crossed with the triploid males were assessed, no visible spermatophore nor sperm were present indicating that G_1 triploid males may be incapable of impregnating females. It is also possible that the triploid males do mate, but that the spermatophores degrade within the female thelycum. The ability of triploid males to contribute to viable spawnings and to impregnate diploid females has never been reported in the literature for any other penaeid species.

The lack of viable spawns from triploid male matings is supported by the gonad histology whereby there was a distinct lack of sperm cells within primary and secondary lumens of the vas deferens compared to a proliferation of these cell types in diploid siblings. Li et al., 2003 also report a lack of sperm being observed in the vas deferens and spermatophores of reproductively mature triploid *F. chinesis.* Spermatozoa present in the testes of *P. monodon* triploids also lacked the rigid spike required to initiate fertilization, which has also been reported for triploid male *F. chinensis* (Xiang et al., 2006). There is no comparative histological data for male triploid *P. japonicus* as triploids produced by stopping the same cell division as in this study and the *F. chinesis* studies being compared here, have never been produced (Sellars et al., 2010). It is worth noting that the histological observations of the triploid and diploid male *P. monodon* gonads at reproductive age (approx. 12.5 months) in this study are similar to those reported for this same family when they were at harvest age (approx. 6 months) (Sellars et al., 2011 in press).

There was no observed difference in the structure of the triploid and diploid male *P. monodon* gonads and spermatophores when observed by the naked eye, however, at closer magnification triploids had no sperm bundles within the vas deferens and spermatophores and no sperm cells within the spermatophores when viewed under light microscopy. The same observations have been reported for *F. chinesis* triploid males (Li et al., 2003).

6.0 Conclusion

Triploid female and male *P. monodon* were incapable of producing viable progeny when crossed with diploids of the opposite sex. Acute reductions in reproductive development of female and male triploid *P. monodon* gonadal tissues were apparent to a point were complete maturation has not occurred. Reproductively mature triploid *P. monodon* were in effect reproductively sterile, indicating that triploidization of this species could provide a means of genetic protection for selectively bred *P. monodon*. Importantly, the practicality and cost-effectiveness of such approaches must be considered in terms of commercial implementation of using triploidy as a means for genetic protection.

7.0 Acknowledgements

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Figure captions

Figure 1. Haemotoxylin and eosin stained sections (10X magnification) of A) a triploid PL_{386} female gonad showing a complete reduction of developing oocytes (open arrows) and incomplete haemal sinuses (shaded arrow), B) a triploid PL_{386} female ovarian lobe with apparently normal connective tissues surrounding and grossly atypical nodule connectives (open arrows), atypical follicle cells (shaded arrows), pycnotic oocyte nuclei (ON), vacuolated cells (VC) and fragmented chromatin; C) a triploid PL_{386} ovary showing few discernable nodules with typical structure, loose connective tissue (open arrow) and few developing oocytes (shaded arrow), and; D) a sibling diploid PL_{386} female gonad showing normal ovarian morphology with prolific numbers of developing oocytes (open arrows) and mature ova (shaded arrows) within the ovarian nodules.

Figure 2. Haemotoxylin and eosin stained sections of A) a triploid PL_{386} male vas deferens (10X magnification) showing a distinct lack of sperm cells (open arrow); B) triploid PL_{386} male seminiferous tubules (20X magnification) showing spermatocytes (open arrows) and a lack of cellular differentiation; C) a sibling diploid PL_{386} male vas deferens (10X magnification) showing prolific numbers of sperm cells (open arrows), and; D) sibling diploid PL_{386} male seminiferous tubules (10X magnification) showing normal cellular differentiation with spermatogonia (open arrows), spermatozoa (shaded arrows) and haemal sinuses.

Figure 3. Reproductive tract from a PL_{386} male A) G_1 diploid and B) G_1 triploid showing a lack of sperm bundles in the triploid. VD = vas deferens, TA = terminal ampule, T = testes, SB = sperm bundles.

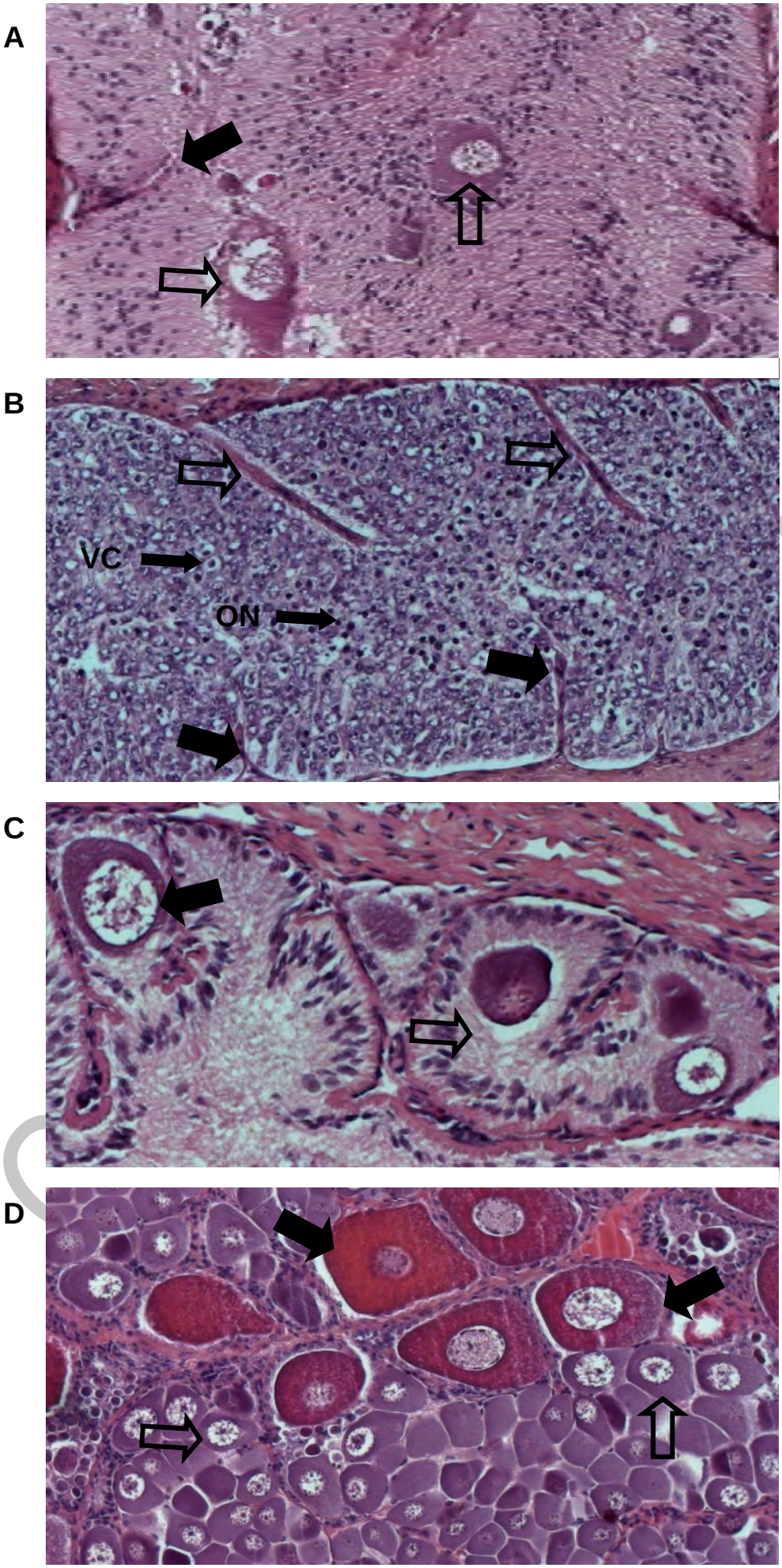

Figure 1.

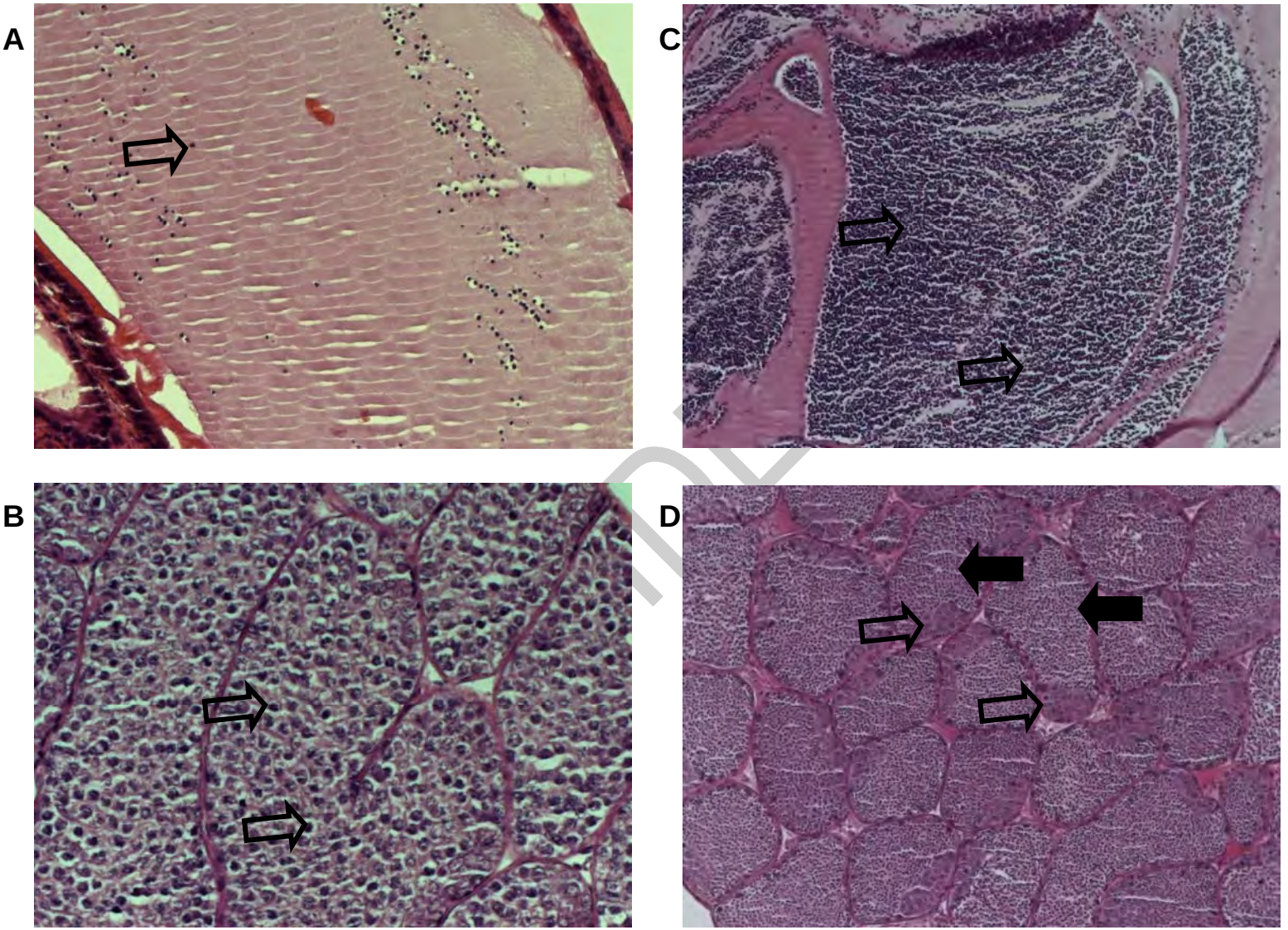

Figure 2.

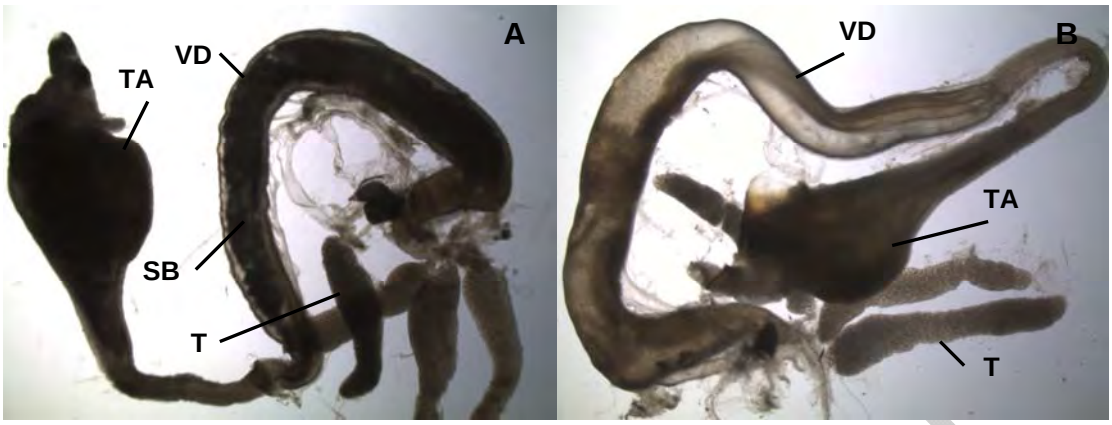

Figure 3.

Tables

Table 1. Experimental crosses used and the number of shrimp stocked when G_1 siblings were PL_{315} and G_0 shrimp were collected from the wild.

	Females		Males	
Tank	Generation / ploidy	Number	Generation / ploidy	Number
1	G_0 diploid	20	G_1 diploid	20
2	G_0 diploid	18	G_1 triploid	18
3	G_1 diploid	11	G_0 diploid	29
	G_1 triploid	8		
	G_0 diploid	10		
4	G_1 diploid	11	G_0 diploid	29
	G_1 triploid	8		
	G_0 diploid	10		

Table 2. Reproductive performance (±SE) of the different triploid and diploid *P. monodon* crosses, excluding the G_1 triploid x G_0 diploid (female x male) cross for which there were no spawnings.

	Cross (female x male)			
Measure	**G_0 diploid x G_1 diploid**	**G_0 diploid x G_1 triploid**	**G_1 diploid x G_0 diploid**	**G_0 diploid x G_0 diploid**
Female weight at 1st spawning	141.50 (8.31) *n=5* nsd	140.33 (3.74) *n=6* nsd	142.41 *n=1* *	143.49 (4.79) *n=10* nsd
Eggs (000's per spawning)	152.34 (8.9) *n=9* nsd	166.10 (21.9) *n=7* nsd	10.45 *n=1* *	159.14 (14.04) *n=10* nsd
Nauplii (000's per spawning)	77.96 (9.97) *n=9* B	0 *n=7* C	0 *n=1* *	57.96 (11.37) *n=10* A
% hatch per spawning	45.65 (7.65) *n=9* A	0 *n=7* B	na	33.02 (7.77) *n=10)* A
% spawnings that hatched	100 *n=9* A	0 *n=7* B	0 *n=1* *	100 *n=10* A
% embryo development per spawning	64.32 (9.92) *n=9* A	0 *n=7* B	na	46.20 (10.76) *n=9* A

n = number of events averaged
na = not applicable
* = excluded from ANOVA as only one replicate spawning
nsd = no significant difference ($P > 0.05$)
Means with different uppercase letters are significantly different ($P < 0.05$) within the 'Measure' parameter (rows)