Increasing seedstock production of domesticated giant tiger prawns (*Penaeus monodon*)

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Non-Technical Summary

2008/756
Increasing seedstock production of domesticated giant tiger prawns (*Penaeus monodon)*

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NON TECHNICAL SUMMARY:
Given the rationale that pond systems are likely the most cost-effective system for large-scale production of *P. monodon* broodstock, this project aimed to determine whether pond-rearing poses a significant risk for broodstock production. The gross reproductive development of males reared in low-density broodstock ponds was found comparable to sibling males reared in controlled-environment tanks. Furthermore, none of the environmental 'stressors' and dietary manipulations examined impacted on male reproductive tract development. Thus, within the boundaries of the parameters tested, we can state that rearing of male broodstock in low-density ponds does not pose inherent risks of gross reproductive tract impairment. Given the typically lower costs of constructing and managing broodstock in large-scale pond systems, as compared to smaller raceway and tank systems, the incorporation of a pond-rearing phase in broodstock production could clearly increase cost-effectiveness of broodstock production at a commercial scale.

Of note, as gross abnormalities in reproductive tract development were not identified in the present project, we have not been able to further knowledge of the likely causal factors which had, on former occasions, contributed to the compromised fertility of pond-reared males.

The project also focused on developing an objective measure of 'male fertility' that could be used commercially; and which could be employed within a monitoring regime. None of the simpler assays, which have typically been used as 'proxy' measures of male fertility, were found to correlate with egg fertilisation rates. Furthermore, none of the assays examined which were performed on spermatophores (and sperm) derived directly from males showed any potential as indicators of male fertility. However, one assay which evaluated levels of 'activation' of 'matured sperm' exposed to 'egg water' (EW-AR assay) correlated with egg fertilisation; and hence provides a reliable measure of male fertility. However, whilst providing an objective measure, this assay is not straightforward to implement commercially; and certainly the applications of this measure are likely restricted. Importantly, an overall approach to long-term monitoring of stocks is suggested; this approach incorporating application of simpler spermatophore/sperm assessments and the EW-AR assay at different points throughout broodstock rearing and at stocking of the hatchery.

During the project a previously undescribed abnormality termed 'hollow sperm syndrome (HSS)' was observed through histology. Whether the presence/prevalence of such abnormal sperm impacts egg fertilisation rates negatively is not yet known; and further research is thus required to establish its commercial importance.

OUTCOMES ACHIEVED TO DATE

A practical regime for monitoring male reproductive development in commercial broodstock systems is proposed. The regime involves evaluations of the reproductive tract of subsets of stocks at regular intervals throughout the rearing phase and more comprehensive evaluations of spermatophores (and sperm) and mating success when stocks are to be introduced into the hatchery for spawning.

Pond systems managed according to current practices, but employing lower rearing densities and a higher quality artificial pellet, provide a cost-effective means for large-scale production of domesticated male broodstock. The duration of the pond-rearing phase will depend on the specific rearing location, but certainly use of ponds for a significant portion of the rearing cycle could significantly reduce costs of broodstock production.

KEYWORDS: male fertility, sperm quality, spermatophore, reproductive tract, reproductive tract development, maturation, egg fertilisation, black tiger prawns

1. Introduction

The aim of this project was to address recurring problems caused by the poor fertility of male *P. monodon* reared in ponds which have slowed progress in up-scaling production of domesticated and selectively bred stocks by the Australian industry. Earlier research projects (e.g. Kenway 2000; Burke et al. 2008; Coman et al. 2008) found reproductive tract development in pond-reared *P. monodon* males was compromised, with spermatophores possessing abnormal gross morphology. Spermatophores from these compromised pond-reared males were generally found to be infertile, as seen through the zero or very low nauplii hatching rates obtained from eggs spawned from inseminated females. This contrasted markedly from the more normal reproductive tract development and spermatophore morphology found amongst sibling males reared in tanks, and that generated significantly higher egg hatch rates (although still well below rates generally obtained with wild broodstock) when females from the same cohort were inseminated artificially with spermatophores of these males.

Reproductive tract anomalies

In some cases where problems with reproductive tract and spermatophore development have occurred in *P. monodon* males reared in ponds, histology has revealed reproductive organs that either seemed immature or when severely compromised were displaying melanisation associated with necrosis at various locations. In other cases, melanisation has been more localised around the gonopore/terminal ampoule region, and ascribed to bacterial infections as a likely

result of exposure to poor pond bottom conditions (Anderson pers. comm. 2007). However, as no evidence of compromised gonad development occurred amongst sibling females reared under the same pond conditions, or amongst sibling males reared in tanks, the phenomenon appeared to be male specific and to date has predominantly been found in green-water pond systems.

The issue is not omnipresent, and reproductive tracts and spermatophores of normal morphology have been found amongst male *P. monodon* reared in ponds for part, or even most of the time required to reach sexual maturity, both in Australia and elsewhere (e.g. Preston et al. 2008; Pratoomchat et al. 1993). The causal factor or factors affecting male reproductive tract integrity are thus not predicated by pond environments per se. However, the manner in which ponds are maintained, flucatuations in temperature and water quality parameters, exposure to environmental chemicals and/or toxins in local supply waters, and to other aquatic species and potential pathogens, makes it difficult to predict the root cause of male infertility, particularly if combinations of such factors are involved.

Previous investigations have not identified any conspicuous factors likely to be the cause of compromised male reproductive tract development. However, they have provided several clues to the kinds of potential factors that might be involved, either partly or wholly. Identifying these factors would provide invaluable information for managing the risk of various husbandry approaches adopted in breeding programs. However, for a range of parameters prone to variation in large-scale broodstock rearing systems, it is also important to know whether males can tolerate exposure to the extremes of these parameters without negative impacts on their reproductive system or sperm fertility.

Sperm fertility

Even in the absence of ‘gross’ reproductive tract abnormalities, or sperm numbers and morphology being within typical ‘normal’ bounds, the egg fertilisation rates and nauplii numbers have been attributed to poor male fertility (Kenway 2000; Coman et al. 2005, 2006, 2007, 2008). However, due to the complex nature of the sperm maturation process in penaeid prawns, including their capacitation within the female thelycum, there remains no easy and reliable means of measuring their capacity to fertilise eggs. Without such methods, and assuming that there are no gross signs of compromised spermatophore or sperm morphology and numbers of normal sperm, the only available means of identifying males with poor fertility is to evaluate egg fertilisation rates following female spawning. However, post-spawning evaluations provide only anecdotal information on male fertility, as female ‘quality’ will also affect egg fertilisation. And clearly, post-spawning evaluations provide no opportunity to influence selections of ‘fertile’ male broodstock for breeding.

Developing a reliable method of quantifying fertility of male *P. monodon* would be invaluable in breeding programs and particularly useful for ensuring husbandry conditions are adequate. It could also be used proactively to select quality males for commercial hatchery production. Moreover, it would be ideal if such a method could be performed easily ‘on-farm’ by skilled personnel. However, more technically involved methods requiring specialised laboratory equipment could still play an

important role in breeding programs, particularly for longer-term husbandry management and monitoring of core breeding stocks.

1.1 Need

With *P. monodon* breeding programs in Australia, there are two broad needs regarding male fertility. The first is to optimise husbandry approaches (i.e. broodstock rearing systems and protocols) that maximise the chances of male broodstock developing healthy reproductive tracts and propagules (spermatophores and sperm). To achieve this it is important to understand what risks are associated with different husbandry approaches and environmental conditions to which broodstock could be exposed. Such knowledge can then instruct what broodstock management protocols are ideal for generating highly fecund broodstock. The second need is a methodology to assess male broodstock quality in relation to propagule quality. It would be ideal if methods to accurately predict male fertility could be applied in commercial settings.

1.2 Objectives

The key objectives of this study were:

- *To examine the effect of environment and diet on reproductive tract and spermatophore development (RT & SD)*
    - To determine whether reproductive tract development in tank-reared males differs significantly (and can be compromised) when prawns are fed on different diets and exposed to different short-term salinity and temperature extremes
    - To determine whether reproductive tract development in males differs significantly (and can be compromised) when males are reared in outdoor ponds at different densities and exposed to different temperature regimes, and when reared in tanks maintained under controlled temperature conditions from juvenile to adult.

- *To develop an objective measure of 'male fertility'*
    - To determine whether sperm count and/or morphology are objective measures (estimators) of male fertility.
    - To develop practical and predictive means to evaluate male fertility that can be used by industry.

An additional objective was included following discoveries made during the course of the project

- *To undertake histology to identify the prevalence of sperm morphology abnormalities amongst wild-caught males and domesticated males reared under different environmental conditions*

2. To examine the effect of environment and diet on reproductive tract and spermatophore development (RT & SD)

Sibling stocks of G1 *P. monodon* were reared under a range of conditions over two seasons and the resulting development of the male reproductive track was assessed. New G1 stocks (i.e. progeny of wild parents) obtained from a commercial hatchery were reared in each season. The first group of experiments focused on comparing male RT&SD of siblings when reared in BIRC ponds at different densities and maintained under different temperature regimes; compared to siblings reared in controlled-environment tanks at CSIRO. The second group of experiments focused on comparing male RT&SD of siblings when reared in CSIRO tanks when fed on different diets and maintained under different temperature and salinity regimes. The aim of these trials was to identify stressors capable of inducing 'gross' developmental abnormalities in the reproductive tract and spermatophores of males. To accomplish this, measures of general health, infection loads of GAV and MoV, reproductive tract and spermatophore morphology and quantitative assessments of sperm numbers and morphology were undertaken at regular intervals during rearing.

2.1 Pond-treatment comparisons & pond versus tank comparisons

Methods

Rearing treatments

In both years, male reproductive tract development was examined in sibling stocks reared in ponds systems at BIRC and controlled temperature tank systems at CSIRO. The BIRC ponds were 400 m^3 in dimension and plastic-lined. Three of the ponds were covered with clear polycarbonate enclosures, allowing some control over temperature in the ponds. A fourth BIRC pond was not covered, allowing no control over water temperature. The uncovered pond exposed stocks to low temperature extremes during the winter period, which was a treatment of interest in the Year 1 trial. The 10 m^3 at CSIRO operated with a continual low water exchange rate that allowed water temperatures to be maintained at a stable 28°C unless part of a differing temperature treatment.

In both years, stocks were reared at differing stocking densities and under differing temperature conditions. Temperature control of the covered ponds, achieved by opening or closing the enclosure doors, was limited and final temperature was still strongly influenced by prevailing climatic conditions. In cooler weather a temperature improvement of 4 to 6°C could be achieved. In the Year 1 trial, the uncovered pond was used to produce natural mid-winter extreme in ambient water temperature with the lowest temperature recorded of 15°C. A full temperature profile of the ponds is outlined in Appendix 1.1. While nominal stocking densities were used in the first year's trial, the actual postlarval stocking densities did vary to a degree due to both postlarvae counting error and to losses incurred within the first few days of pond stocking.

The age at which prawns were exposed to temperature stresses was aligned to that experienced typically during rearing of domesticated broodstock in North-eastern Australia to accommodate a spring spawning cycle. Low temperature stress commenced at a prawn age of approx. 6 months, when prawns spawned in spring would normally start to experience winter water temperatures. High temperature stress commenced at a prawn age of approx. 4 months when prawns spawned in spring would typically experience summer water temperatures. The different pond and tank rearing parameters examined in each year of the study are summarised in Appendix 1.2. Whilst these trials provided some opportunity to compare different rearing conditions, they allowed the critical comparison of pond versus tanks rearing of siblings, which was considered as important as the inter-pond comparisons. Prawns reared in tanks were fed a basic diet (of high quality/high protein Kuruma pellets) to maintain accordance with stocks reared in ponds, and a tank diet comparison is detailed in a later section.

Evaluations

Male and female prawns were weighed at regular intervals for purposes of pond management, but also at the various sampling points in both ponds and tanks. General body condition was also scored at each sampling point based on levels of lesions, necrosis and other shell markings. Evidence of females having mated was determined by examining the thelycum for presence of whole or remnants of spermatophores.

Samples of haemolymph were collected from all male and female prawns soon after transport to the laboratory (at BIRC or CSIRO respectively) by heart puncture, to determine its refractive index/osmolality as an indicator of protein content and condition. The moult stage of each prawn was recorded, as this is known to influence refractive index and osmolality. Gill pieces were collected into ethanol for determining infection status and loads of GAV and MoV either by RT-nested PCR or real-time quantitative (q)RT-PCR.

Spermatophores were electro-ejaculated from a subset of males and weighed. One of the twin spermatophores was placed in seawater to allow the tail-fan (i.e. wing) to unfurl to score its gross morphology. Criteria used to assess the gross morphology (i.e. physical condition) of spermatophores, included fullness (i.e. opaqueness of the sperm bundles), colour, form and presence/absence of a tail-fan. The other twin spermatophore was processed to estimate total sperm number per spermatophore, % normal and abnormal sperm and ratios of sperm number and normal sperm to spermatophore weight.

Reproductive tracts (i.e. testes, vas deferens, terminal ampoule and the developing spermatophores within the tract) were dissected from most males to assess for gross morphological abnormalities and to fix samples, when necessary, for subsequent histological evaluation. Head tissues of male prawns were processed for histology. Microscopic evaluations focussed on male gonad tissues including the testes, spermatogonial cells, vas deferens, sperm masses in the vas deferens, and the sperm cells at various locations along the tract. In later trials histological evaluations also employed transmission electron microscopy (TEM) to provide more detailed information on sperm ultrastructure.

Histology was also used to examine other tissues of both males and females from the different ponds and tanks. The hepatopancreas was examined to determine relative lipid content, as this is a good indicator of dietary intake and general health. The lymphoid organ was examined to assess levels and types of spheroids, as this is a general indicator of viral infection and infection severity. Gill, heart, muscle, haematopoietic tissue, eye and exoskeleton structure were also examined to derive an overall picture of the general health and condition of prawns.

Results

Growth & survival

In Year 1, prawn growth rates in the three covered ponds, regardless of stocking density, were similar to that seen in tanks. Growth slowed significantly in the uncovered pond in conjunction with cooler seasonal water temperatures. Prawn survival in tanks was high (>95%). Survival in ponds could not be determined accurately, however mid-cycle population estimates using feed consumption and proxy measures of final harvest numbers indicated that the covered pond stocked at 8 m^{-2} (Cov Pond - 8m^{-2}) suffered higher mortality than the other ponds during the earliest phase of grow-out due possibly to a bloom of colonial hydroids. The uncovered pond (Uncov Pond - 8m^{-2}) suffered high late-cycle mortality that coincided with a spike in viral loading during the cooler months. Estimates at harvest suggested that the other two covered ponds had reasonably high survival rates (>70%).

In Year 2, prawn growth rates in the covered ponds were again similar to their siblings reared in tanks. Notably, pond stocks grew faster in Year 2 than in Year 1 trials, most likely due to the timing of PL stocking, which occurred earlier providing a more extended period of warmer temperatures at the end of summer. Elevated temperatures induced in one covered pond (Cov Pond - heat exacerbated) (see Appendix 1.1) appeared to have little observable impact on growth of male prawns. Prawn survival in tanks was again high (>90%). Prawn survival in the control pond (Cov Pond - control) was estimated to be lower than in the treatment pond (Cov Pond - heat exacerbated), seemingly due to pond-specific factors as the mortalities occurred very early during grow-out before elevated temperatures were induced. Of note, sexual dimorphism of weight was more pronounced in the control pond (Cov Pond - control), with final mean weights of females (81.3 g) being much higher than females reared in the heat-exacerbated covered pond (69.0 g). In contrast, males in these two ponds were comparable in mean weight (47.2 g vs 47.4 g). The reason for this differential dimorphism between ponds is not clear; but may have been associated with the differential survivals in the ponds.

Body condition

In Year 1, the tank-reared prawns generally had higher levels of shell damage earlier in grow-out, but their general appearance improved over time to be comparable to siblings reared in the covered ponds. Notably, whilst the condition index of prawns reared in the uncovered pond (Uncov Pond - 8m^{-2}) was comparable to the other ponds through much of the grow-out period (i.e up to 5 ½ months), it dropped below that of prawns reared in the covered ponds or tanks by mid-winter (9 months) when prawns had been exposed to low water temperatures for some time. Despite this, no statistical

differences in body condition indices were found amongst the pond or tank prawns. In Year 2, the shell condition of both pond and tank prawns was typically high throughout the entire grow-out period.

Mating

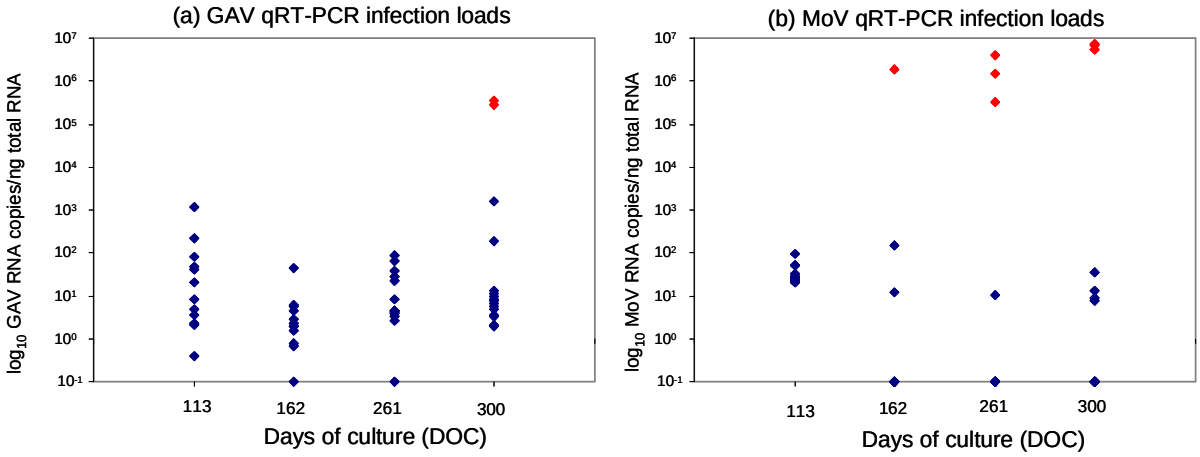
Impregnation of females with spermatophores was monitored over Year 1 rearing to estimate the earliest age of mating. The earliest evidence of females having mated, as determined by thelycum dissection, was at approx. 5 ½ months in one of the covered ponds. Evidence of mating was common in ponds and tanks by 8 months. In the Year 2, dissections confirmed a high incidence of female mating was commonplace by 7 months.

Haemolymph measure and viral prevalence

In Year 1, the refractive index of haemolymph collected from prawns was typically similar amongst siblings reared in the covered ponds and tanks. Notably, the mean refractive index was lower in prawns sampled from the uncovered ponds at the final sampling point most likely due to the prawns having experienced cooler water temperatures for some time that reduced feed intake. Haemolymph osmolality varied little amongst prawns from any of the ponds but notably was somewhat lower amongst tank-reared prawns at the final sampling point. In Year 2, no major differences in osmolality were detected amongst prawns sampled from any of the ponds or tanks.

In Year 1, GAV was detected by real-time RT-PCR at very low infection loads in most prawns sampled from either ponds or tanks at any of the sampling points. However, following the transfer of a subset of the stocks from each of the 4 ponds at 9 months for over-winter rearing in tanks at CSIRO, GAV infection loads were found to be higher in prawns originating from the uncovered pond (Uncov pond - $8m^{-2}$) when assessed at 10 months (i.e. 300 DOC) yet remained low in prawns originating from the 3 covered ponds (Figure 1). MoV infection was either not detected or detected at low levels approaching the sensitivity of test at most sampling points, with the exception of prawns originating from the uncovered pond (Uncov pond - $8m^{-2}$). Amongst prawns sampled from this pond, high-level MoV infection was detected in 1 of 3 prawns assessed at 5 ½ months (i.e. DOC 162) and in each of the 3 prawns sampled at 2 later sampling points at 9 and 10 months (i.e. DOC 261 and 300) (Figure. 1). Amongst prawns originating from the 3 covered ponds, MoV was not detected in most prawns at 10 months (i.e. 300 DOC), and when detected, infection loads were low. In Year 2, GAV was either not detected, or detected at only very low infection loads in most prawns reared either in ponds or tanks, while MoV was not detected in any prawns sampled at any of the sampling points.

Figure 1
Infection loads *(log_{10} copies of virus per ng of RNA)* of (a) GAV and (b) MoV in the *Penaeus monodon* over time in the four different pond stocks. The data points in red identify the prawns sampled from the uncovered pond (Uncov pond - $8m^{-2}$) found to be infected at high levels with GAV and/or MoV, while the data points in blue identify prawns from pond and the covered ponds for which infections loads were considered low or were not evident.



Gross morphology of the reproductive tract and spermatophores

The three major components of the male reproductive tract, testes, vas deferens, terminal ampoule, were dissected and observed for males from all ponds at the earliest sampling point at 2 months. As might be expected due to their lack of maturity, no sperm bundles were evident in the vas deferens and few testes lobes had formed. By 4 months, males sampled from all ponds and tanks showed sperm bundles in the vas deferens, and higher numbers of testes lobes and spermatophores with normal morphology containing sperm of normal morphology. Dissections of the reproductive tracts from males reared in ponds and tanks in Year 2 similarly found no evidence of gross abnormalities at any stage.

Spermatophore measures

In Year 1, mean spermatophore weights of pond-reared males increased dramatically from 3 to 5 ½ months (i.e. 113 to 162 DOC; Appendix 1.3a). Mean weights had increased further after 9 months (i.e. 261 DOC) in males reared in the 3 covered ponds but not amongst males reared in the uncovered pond. No significant differences in mean spermatophore weights were found between samples from the covered ponds and the tanks at comparable times. Spermatophore weight: body weight ratios increased amongst all groups of pond-reared males from 3 to 5 months, but typically plateau-ed between 5 ½ and 9 months in both the pond and tank males.

Mean spermatophore weights were higher in Year 2 than Year 1. Notably, due to the higher mean body weights in Year 2, the resulting male spermatophore weight: body weight ratios were similar across both years. Mean spermatophore weights and spermatophore weight: body weight ratios were comparable in siblings reared in tanks and covered ponds.

The general physical presentation (fullness, colour, form and presence/absence of a tail-fan) of spermatophores was largely consistent across the various pond- and tank-reared males examined in either year. Spermatophore fullness was observed to be a little lower amongst Year 2 males compared to Year 1 males despite the Year 2 males being slightly larger in size.

Sperm measures

For the range of sperm parameters assessed in Year 1 (Appendix 1.3b), including number normal, % normal, normal sperm: spermatophore weight ratio), little variation was evident amongst males reared in the various tanks and covered ponds. However, males reared in the uncovered pond (Uncov Pond - $8m^{-2}$) had much lower average sperm counts and % normal sperm at the first sampling point, most likely due to their smaller size and belated maturity. Despite this, no significant differences in any spermatophore measure were evident across the whole rearing period amongst Year 1 pond or tank stocks.

Sperm numbers and density (i.e. the number of sperm per mg spermatophore weight and per gram body weight – see also Appendix – 1.3c) were lower for Year 2 compared with Year 1. Whilst sperm densities amongst males from the control pond (Cov Pond - control) dipped abruptly at the final pond-sampling point at 7 months, no other significant differences in sperm numbers or related parameters were evident amongst control pond, treatment pond and control tank males sampled across Year 2.

In Year 2, high numbers of abnormal sperm were evident amongst males at the initial sampling of the ponds at 3 months, despite these males being larger than the males in Year 1 at a comparable time. Notably, in Year 1, abnormal sperm were only evident in smaller males. Lower sperm counts across the Year 2 males at the early sampling point were accompanied by sperm abnormalities in the testes, which were not noted in the previous year, and these abnormalities persisted to a higher degree in the tank-reared males.

Histological observations

In both Year 1 and Year 2, histology on the male reproductive tract identified minor incidences of localised melanisation amongst a few of the pond- or tank-reared stocks. The melanisation was evident only in a very small proportion of males, and there was no consistent pattern of its appearance.

In Year 1, lipid stores in the hepatopancreas were generally higher in tank-reared compared to pond-reared males; however lipid stores were sufficiently high in both pond and tank stocks to indicate all stocks were in good nutritional health and not stressed. No consistent link was apparent between lipid storage levels and any other measure of male reproductive tract condition including the prevalence of abnormal ‘hollow‘ sperm. Low-grade spheroidosis, indicative of only ‘low-level’ viral infection, was evident in lymphoid organs of most males sampled either from ponds or tanks in either year. However, in Year 1, males sampled from the uncovered pond (Uncov pond - $8m^{-2}$) at the later sampling points after exposure to cooler pond water did display higher-grade spheroidosis suggestive of higher-level viral infection. Notably, no association between the sperm nuclear ‘abnormalities’ and spheroidosis

severity were evident, as the abnormalities occurred even in males with the lowest grades of spheroidosis.

The discovery of this 'hollow' sperm syndrome (HSS) characterised by sperm devoid of nuclear material in the testes and vas deferens of both pond- and tank-reared males was a major outcome of this study as it has not been described previously (discussed in more detail in a following section).

2.2 Tank-treatment comparisons

Methods

Rearing treatments

In tank experiments conducted at CSIRO in each year of the 2 year project, male reproductive tract development was examined after stocks had been exposed to specific environmental and dietary manipulations. The manipulations involved short-term exposure of prawns to various combinations water temperature and salinity, and long-term feeding on diets varying in levels of fresh-frozen ingredients and artificial pellets.

Prawns were typically reared in 10 m^3 tank systems with sand bottom substrates and flow-through heated water, until (and following-on after) defined environmental stresses were applied by transferring subsets of prawns into other tanks. Based on needs, the tank systems used for applying the 'stress treatments' varied between trials (e.g. static water systems were needed for salinity trials). As in pond trials, the ages at which prawns were exposed to each stress were aligned to temperature scenarios typical for broodstock rearing to meet spring spawning cycles. Consequently, the low temperature stress was commenced when prawns reached an age of approx. 6 months (a typical age on entering winter) and the high temperature stress was commenced at an age of 4 months (a typical age encountered mid-summer).

Potential environmental stresses that were mimicked and the diets which were evaluated, often in various combinations, are listed below:

- Low temperature: 19°C for 5 days or more
- High temperature: 35°C for 5 days or more
- Control temperature: 28°C-29°C

- Low salinity: 10‰ for 5 days or more
- High salinity: 50‰ for 5 days or more
- Control salinity: 35‰-36‰

- Artificial and fresh-frozen invertebrate diet (Art Fr)

- Artificial commercial pellet diet fed (Art)
    - Artificial diet was Lucky Star *P. japonicus* grower pellets
    - Frozen invertebrate diet comprised squid and green lipped mussel
    - Dietary treatments were maintained for the duration of the experiment

Two control groups were run simultaneously alongside each stress group. One control involved comparable prawn handling but without being subjected to the potential stressor, while the second control involved no handling, with prawns maintained in their primary rearing tanks. Representative prawns were evaluated prior to the stress period, and representatives of each stressed and control group were evaluated at various points after the stress event. The groups fed on different diets over the entire rearing period were also evaluated at various points during rearing as appropriate.

The specific details of the two experiments are summarised below.

Low temperature-diet experiment

- 6 combinations of 2 dietary, 2 temperature, and 2 handling treatments
- Effects were evaluated 2 ½ months after the short-term temperature stress at 6 ½ months

High temperature – salinity experiment

- 6 combinations of 2 temperature and 3 salinity treatments, as well as a non-handled control group
- Effects were evaluated approx. 2 ½ months after the short-term temperature-salinity stress at 4 months

The different stress treatments applied in each year of the study are summarized in Appendix 1.4. Temperature and salinity profiles used during the various stress events are summarized in Appendix 1.5 and 1.6.

Evaluations

Quantitative assessments of reproductive tract development in male prawns stressed in tanks were performed as described above for the prawns stressed in ponds.

Results

Low temperature-diet experiment

Growth rates were higher amongst control prawns fed on the control diet (Cont-Art Fr) than control prawns fed solely on the artificial diet (Cont-Art). No significant differences in body weight were evident between prawns in ‘temperature control’ and ‘low temperature’ groups fed on either diet, or between ‘handling’ and ‘temperature’ groups. Prawn survival was high (>90%) and unaffected by any treatment examined. Prawn body condition improved after the initial measurement point at 3 months and thereafter was unaffected by any dietary and stress regime. Due to an experimental design that necessitated crossing of different treatment groups post-stress in long-term holding tanks, it was not possible to evaluate the effects of

low temperature on mating. However, the effects of diet on mating patterns could be examined. Averaged for all females across the different 'temperature-stress' treatments at 6 ½ months, evidence of females having mated was comparable in both dietary groups (38% control diet and 44% artificial diet).

Neither abnormalities, nor differences in development or gross morphology were evident amongst the tracts of any of the males examined from any of the experimental treatments 'post-treatment' exposure at 6 ½ months. Furthermore, no differences in mean spermatophore weights were found between dietary–stress treatments at 6 ½ months. The ratio of mean spermatophore weight: mean male body weight (%SSI – percentage spermatophore: somatic weight index) was highly consistent among stress treatments in the control diet treatment groups. There was greater variation of the sperm ratios for the artificial diet treatment group but the differences were not significant.

No difference in sperm numbers was found between the low temperature and control temperature treatment at 6 ½ months for either diet. Sperm counts were significantly higher for the control diet treatments than the artificial diet treatments when analysed across all temperature treatments. Percent normal sperm did not differ between treatments.

Histology revealed that male reproductive tract development was largely normal across all treatment groups. Observations of sperm abnormalities were spread across males from all groups.

High temperature – salinity experiment

Survival and body conditions were consistently high and no significant differences in male weights were evident across the handling-temperature-salinity treatments. As in the low temperature - diet trial, the experimental design did not allow the effects of temperature and salinity stress on mating to be evaluated rigorously. However, the majority of females (>70%) sampled from the range of stress-treatments examined were found to have mated when assessed at approx. 6 ½ months (i.e. 2 ½ months 'post-stress').

No qualitative differences in reproductive tract maturity or normality were evident amongst males examined from any treatment group at 6 ½ months.

Spermatophores of males sampled from all treatment groups were typically normal in gross morphology. No significant differences in spermatophore weight and % SSI were found between dietary-stress treatments.

While quite variable, no significant differences in total sperm number or total sperm number per mg spermatophore weight were evident amongst males sampled from the different treatment groups. Notably, spermatophores from the 'control' treatment (i.e. 28 & 36) males exposed to the same handling as the temperature-salinity stressed males produced the fewest sperm on average. Certainly there was no evidence of sperm production being reduced by short-term exposure to higher temperature, lower salinity, or higher salinity water conditions (or any combinations of these parameters).

As in the low temperature - diet trial, histology revealed that male reproductive tract development was largely normal across all treatment groups.

2.3 Observations from males sampled from commercial ponds

Rationale

Due to the absence of evidence of grossly impaired reproductive tracts or spermatophore development (RT & SD) in project experimental groups, it was decided to undertake a limited survey of *P. monodon* males sourced from commercial ponds, which were potentially exposed to more extreme rearing conditions. The ponds from which males were sampled were chosen because, according to the pond manager, they had experienced at least one of the following: (i) adverse environmental stressors over the grow-out cycle (ii) poor prawn survival suggestive of stress events (known or unknown) having occurred (iii) displayed poor general prawn condition including a high incidence of brown gills, dirty/brown genitalia and external necrosis and damage to antennules, rostrums, pleopods and periopods.

As the rearing conditions in these commercial grow-out ponds were not optimised for broodstock maturation, the hypothesis was that RT&SD problems might be more evident, thus affording useful additional information of the nature of the syndrome.

Methods

Ten male *P. monodon* were sampled from 6 ponds viewed by farm managers to have performed poorly in terms of prawn survival as well as prawn growth rates and general condition. The external physical condition of the males, including genitalia, and of spermatophores electro-ejaculated (morphology, colour, fullness) was assessed pond-side. A group of 50 males collected from 5 of the ponds was transferred to controlled environment tanks at CSIRO to examine their subsequent survival and RT & SD following a period of rearing in controlled temperature clear-water systems. After being reared for 1 ½ months (up to an age of approx. 6 ½ months), a subset of the remaining males was sampled for either histology or RT & SD (following methods described earlier).

Results

‘Pond-side’ evaluations of spermatophores ejaculated from the males identified that the majority (64%) were normal in colour and general morphology. Spermatophore fullness varied with approximately 58%, 35%, and 7% being classified as 50%, 75% and 100% full, respectively. The prevalence of spermatophore abnormalities varied considerably between ponds ranging from 11% to 88%.

Amongst the 50 males collected from the commercial ponds and on-reared in tanks to 6 ½ months of age, survival was very poor, with only 9 (18%) surviving. The general condition of these surviving males was poor, with all but 2 displaying necrosis and varying levels of appendage damage.

The spermatophores from three of these males were assessed 'tank-side'. All three had abnormal spermatophores, with the morphological abnormalities including absence of 'tail fans' and discolouration (ranging from light brown to brown).

Eight of the males were assessed for GAV and MoV infection load, while six 'heads' and three ejaculated spermatophores were processed for histology. Moderate to high GAV and/or MoV infection loads were evident in all sampled prawns, which thus might have contributed to the high mortalities. Histological evaluations identified no obvious reproductive tract abnormalities in any of the six males examined and only one male showed signs of minor degeneration of spermatophore material in the vas deferens. Hepatopancreas lipid storage levels amongst the males was typically moderate (60-70%) and Type 1 and 2 spheroids were prevalent (>60%) in the lymphoid organs of all prawns consistent with evidence of moderate/high infection loads of GAV and MoV. Where observed in the vas deferens, sperm was typically 'normal' in morphology and only a few abnormal sperm were identified in the vas deferens of one male and the testes of two males.

2.4 Discussion: To examine the effect of environment and diet on reproductive tract and spermatophore development (RT & SD)

No significant effects on male *P. monodon* RT&SD surfaced in any of the pond- and tank experiments undertaken as part of this project, regardless of the nature of the environmental or dietary stressor/s applied. Indeed, RT&SD was mostly comparable, and sometimes slightly superior, for pond-reared males than sibling males reared in controlled environment tanks. Moreover, these findings of normal male RT&SD in research stocks are consistent with what has been observed recently amongst stocks of domesticated males reared on-farm in low-density broodstock ponds for most of their maturation cycle. In fact, the only males in which RT&SD was compromised to 'some extent' was a group of G3 domesticated *P. monodon,* which were not used in the present experiments, that had been reared solely in tanks, lending support to pond-rearing *per se* not being a prerequisite for RT&SD abnormalities.

It is worth noting that moderate levels of spermatophore abnormalities were evident amongst males reared in commercial grow-out ponds in which moderate health problems were occurring toward the end of the production season. As might be expected, the environmental conditions in these commercial ponds were not as conducive to maximal male RT&SD as low-density broodstock rearing ponds. Notably, the specific causes of these elevated levels of spermatophore abnormalities in these commercial stocks could not be deduced, and most likely arose due to a combination of sub-optimal and interacting/compounding factors. Certainly, the abnormalities found in these pond males were not completely reversed after their rearing in controlled-temperature clear-water tank systems for 6 weeks; and such inabilities to rectify compromised RT&SD has been noted in earlier studies.

Whilst not identifying the causes of compromised RT&SD, the present study is instructive on what environmental parameters are tolerated by male *P. monodon*. Of potential significance, infection loads of GAV and MoV estimated by PCR and lymphoid organ spheroidosis severity were generally low and thus unlikely to have impacted the overall health of males. However, even amongst the single group of

pond-reared *P. monodon* exposed to cooler waters over winter in which high MoV loads established, these infections did not overtly impact male RT&SD despite their external and physiological condition being much poorer than pond-reared males not exposed to cold water temperatures and not infected at high levels with MoV. As noted above the only group of males in which spermatophore development was compromised moderately were those reared in commercial ponds under conditions that are considered 'far from ideal' in terms of broodstock rearing. As water quality parameters and microbial ecosystems experienced by males reared in these commercial ponds were not evaluated, one can only speculate as to what the potential causal factors were.

Given that the parameters assessed specifically in this project did not compromise male RT&SD, it seems that rearing of *P. monodon* broodstock in 'typical' broodstock rearing systems carries no inherent risk to male reproductive development; the caveat being that broodstock rearing conditions are within the scope (level and duration) of those assessed in this study. Certainly, we can not rule out that more extreme levels of the parameters assessed, or different combinations of these parameters, may expose the males to higher risks of compromised RT&SD. However, given the wide range of the parameters trialled and the negligible impact found on male RT&SD, it seems that most 'normal' environmental fluctuations to which the broodstock will likely be exposed in broodstock rearing systems should not risk male maturation. Certainly, this finding provides encouragement that ponds are a viable option for rearing *P. monodon* for significant parts of their rearing cycle. Given the high costs of maintaining smaller raceway and tank rearing systems, it does seem that inclusion of a pond-rearing phase could significantly increase cost-effectiveness of broodstock production, and allow for up-scaling of broodstock production.

Notably, as the causative agents impacting on compromised male RT&SD were not elucidated, and as these agents are potentially ephemeral in nature with potential to enter rearing systems without warning, it is strongly suggested that core breeding stocks are reared at multiple locations; preferably with high degrees of environmental separation (e.g. separate water sources). Such biosecurity through rearing system replication, coupled with a monitoring program for early warning of compromised male RT&SD, currently provide the best insurance against the potential impact of compromised male RT&SD; while at the same time providing broader insurances against loss of core stocks due to significant disease episode or system failure.

3. To develop an objective measure of 'male fertility'

3.1 Quantifying the correlation between sperm count, morphology and egg fertilisation

Methods

A novel approach was used to examine the relationship between numbers and morphology of sperm derived from male spermatophores and subsequent rates of egg fertilisation following spawning. The approach involved estimating the sperm count/morphology patterns in spermatophores from different males, followed by insemination of 'different' spermatophores into single females. The resulting contribution that each male/spermatophore made toward subsequent egg fertilisation of the female at spawning was assessed by genotyping the embryos following spawning; and this allowed the relationship between sperm count/morphology and egg fertilisation to be determined.

The experiment was undertaken in this manner to standardise for any female-based influences on fertilisation so that any male influences could be identified more easily. Methods were established to (i) accurately estimate the number and morphology of sperm derived from the twin of the inseminated spermatophore, (ii) artificially inseminate females reliably with spermatophores and (iii) accurately assign male parentage based on genotyping of DNA isolated from individual embryos and nauplii. Egg fertilisation rates were determined for 22 females that spawned. Subsamples of the fertile embryos were collected from these spawns, as well as tissue sampled from the potential parents, were genotyped. Total sperm number and the number of normal sperm estimated for each male were plotted against estimated percentages of eggs fertilised by each male as determined by genotyping.

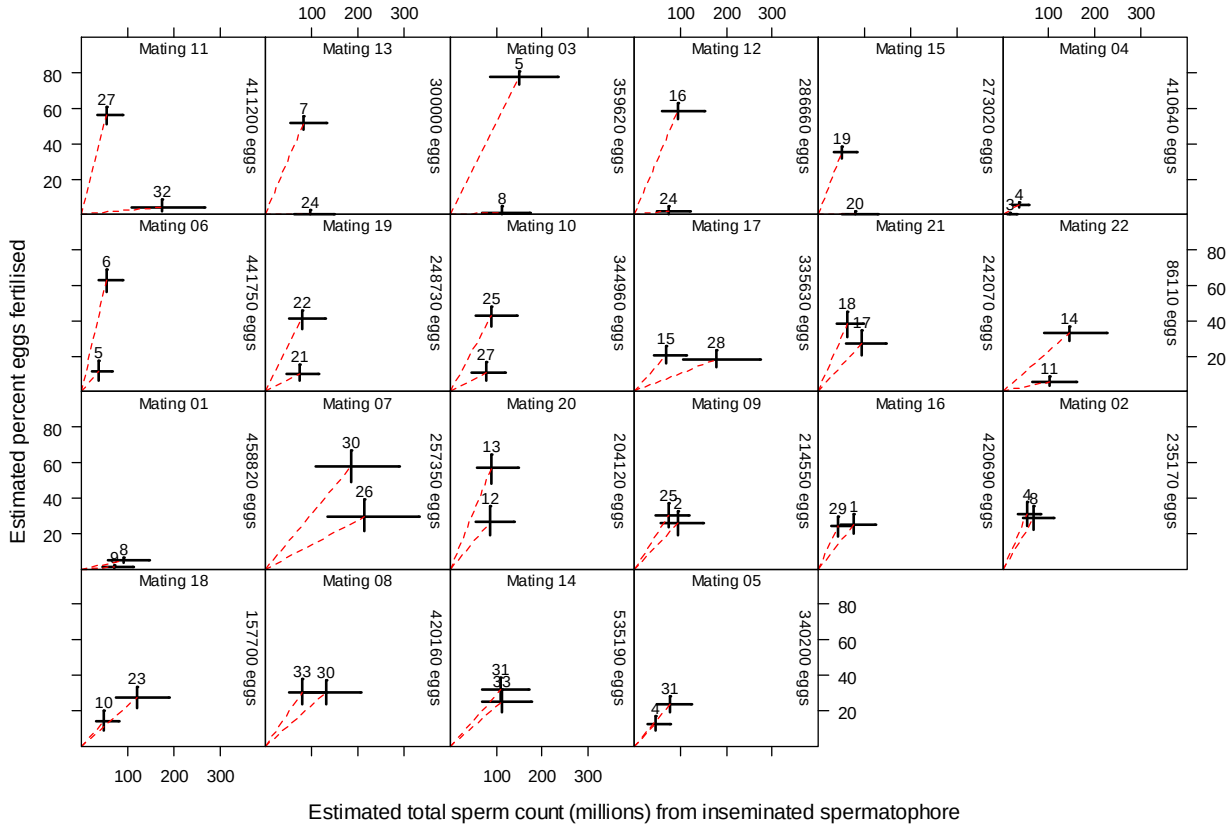
Results

Our data showed that egg fertilisation is not related directly to either sperm number or the number of sperm with normal morphology. In 9 of the 22 batches of eggs spawned from females inseminated, one male fertilised a substantially greater number of embryos than the other male, and this did not necessarily correlate to which male had higher sperm numbers (or higher numbers of normal sperm). This is best demonstrated with spawnings from females in mating pairs 3, 11, 12, 13 and 15 (Figure 2) where sperm from one male contributed to little if any of the fertilised progeny, with fertilisation estimates below 3.5% of total egg numbers even though sperm numbers ranged from 64.2 to 189.1 million. These low fertilisation rates were not caused by problems with ability of eggs to be fertilised as sperm from the other male were determined to have fertilised between 35.6% and 79.2% of total egg numbers. Furthermore, 3 of the males that fertilised almost no progeny had higher estimated sperm numbers and sperm with normal morphology than the male which fertilised most of the progeny.

The results suggest that factors other than number and morphology make major contributions to the ability of sperm to fertilised eggs. Consequently, these basic

sperm characteristics at best provide only crude measures of male fertility, and are of limited value in estimating the fertilisation capability of males at least when gross spermatophore morphology is normal and sperm numbers are not abnormally low.

Figure 2
The estimated total sperm number (millions) from inseminated spermatophores and estimated percent of eggs fertilised for each male within 22 separate matings, whereby one female was artificially inseminated with two spermatophores, one each from two different males. The numbers identify the individual males that were used in each mating.



The absence of egg fertility correlations found has provided impetuous to evaluate whether other measures of sperm quality might have potential to predict its egg fertilisation capability. The results, however, indicate that egg fertilising rates are likely to vary markedly from male to male even when spermatophores and sperm numbers and morphology assessed for representatives of groups of males are comparable. While this is, to a large degree, an assumption that we have made, we consider it reasonable given our findings. Consequently, when examining future potential measures of sperm quality, any measures trialled that were not able to discriminate between individual males (i.e. measures for which no variation was found between males) was assumed to be unable to predict the ability of an individual male to fertilise an egg, and therefore not to have potential to estimate sperm quality.

3.2 Assessing alternative measures of male fertility from male spermatophores (excluding histological evaluations)

Methods

Approaches to assessing male fertility were examined using spermatophores ejaculated from males that thus contained sperm not yet 'capacitated' through maturation within the thelycum of females. Each approach was assessed initially for its ability to discriminate sperm quality characteristics of different males from a broad spectrum of sources. When approaches were found to be incapable of detecting any differences, experiments were not progressed to examine for associations with egg fertilisation. Methods examined focused on quantifying differences in various physiological, chemical and/or structural characteristics of sperm cells.

Methods using stains for assessing sperm membrane integrity (SYBR14/Propidium iodide) and mitochondria functioning (Rhodamine-123/PI) were optimised for application to *P. monodon* sperm. Optimisation was needed for cell staining processes and quantification using fluorescence-activated cell sorting (FACS). The Rhodamine 123/PI staining protocol was tested on sperm from 9 males and the SYBR-14/PI staining protocol was tested on sperm from 7 males.

UV spectrophotometry (Nanodrop® spectrophotometer) and agarose gel electrophoresis were used to obtain crude measures of sperm DNA quantity and quality. To undertake male comparisons, methods were devised to standardise sperm samples, extract DNA sufficient in quantity and purity for analysis and ensure appropriate sample replication for statistical validity. The accuracy of the methods in quantifying DNA was assessed by examining DNA extracted from sperm diluted to cell densities of 30, 20 and 10 million/mL from males of different origins.

A 'Single Cell Gel Electrophoresis' assay (Comet assay) was customised so that it could be applied to *P. monodon* sperm cells. This involved each step of existing Comet assay protocols being refined systematically using cells most likely to possess similar characteristics to prawn sperm cells (e.g. oyster sperm cells and prawn haemocytes). The customised assay was validated by quantifying increases in DNA damage levels in sperm exposed to UV light for increasing durations. The Comet assay was then used to quantify DNA damage levels in sperm derived from spermatophores of wild and third generation (G3) domesticated *P. monodon*.

Results

Evaluations of the membrane integrity of sperm released from ejaculated spermatophores showed that it was predominantly viable (i.e. intact membranes) and that the percentages of viable sperm (95.5% to 99.7%) varied little amongst the males assessed. Evaluations of mitochondrial function supported high viability of the sperm (i.e. functioning mitochondria and intact membranes) again with little measured variation (98.4% to 99.5%) amongst the males examined. Based on the premise that overall sperm ability to fertilise eggs will vary amongst males, the staining methods

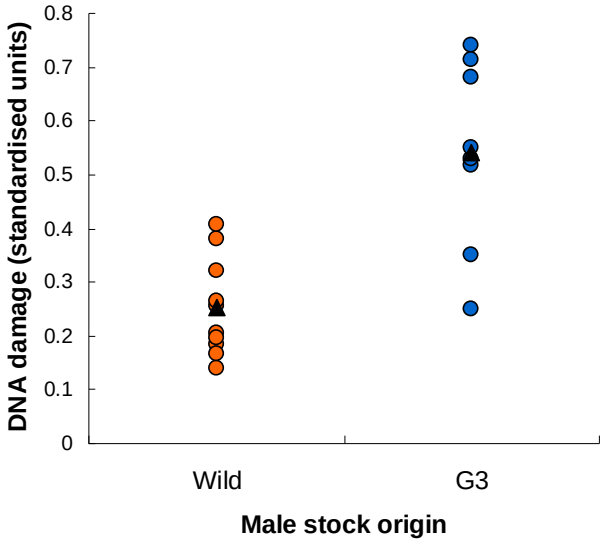
used to assess membrane integrity and mitochondrial function are not capable of quantifying this in *P. monodon*.

The quantity of DNA in sperm was also examined as a sperm quality measure. DNA was extracted successfully from sperm suspensions and quantified using a Nanodrop® UV spectrophotometer. The data indicated that at least 2×10^7 sperm are needed to quantify DNA reproducibly. DNA purity as determined from A_{260nm}/A_{280nm} absorbance ratios being mostly above 1.8 (range 1.74 to 1.87) was high. Whilst differences in DNA quantity were detected amongst sperm collected from different males, there were other problems associated with this measure including: DNA quantity did not correlate linearly with sperm density; inconsistencies occurred between sperm released from either of the 2 spermatophores; and measurement variability was high. Sperm DNA quantification was, therefore, not useful for quantifying sperm quality of males.

Crude assessment of the integrity of DNA extracted from sperm by agarose gel electrophoresis also failed to identify any meaningful differences amongst sperm from different males, nor was this measure able to identify differences between sperm exposed to UV irradiation for differing durations. The method was deemed to be too crude to measure sperm DNA quantity or quality accurately and thus not useful for assessing overall sperm quality.

The Comet assay was able to reliably identify increases in DNA damage induced by increased exposure to UV irradiation. The protocol was highly reproducible with little variation detected amongst replicate samples of the same sperm. Sperm DNA damage (expressed in customised standard units ± S.D.) was higher for domesticated G3 broodstock (0.54 ± 0.17) than wild broodstock (0.25 ± 0.09) (Figure 3). However, given that the maximum score for DNA damage is 4.0, it was evident that males from both sources had low levels of DNA damage. Amongst the G3 males, 54% of sperm cells were assessed as having no DNA damage, and the majority (39.6%) of cells containing damaged DNA were Graded 1 (<25% DNA damage). Amongst the wild males, the majority (77.1%) of sperm cells had no DNA damage, with 20.8% sperm with damaged DNA categorised as Grade 1. Of note, the G3 males had spermatophores displaying some abnormalities including the absence of tails, slight discolouration and aberrant morphology. Despite these spermatophore abnormalities, the finding of over 50% of sperm with intact DNA suggests that *P. monodon* sperm DNA is fairly robust.

Figure 3.
DNA damage in sperm from individual wild and third generation (G3) domesticated broodstock. The ▲ represents the mean DNA damage for each stock. The standardised units shown on the Y-axis describe the mean proportion of cells per each of the visual Comet scores (i.e. ranging from 0-4).



3.3 Assessing alternative measures of male fertility from sperm post-maturation within the female's thelycum

Methods

Approaches to assessing male fertility were also examined using sperm taken after a period within the female thelycum, these sperm cells were therefore matured/capacitated at the point of conducting the assay. For such assays, it was possible to examine the relationship between the assay outcome and 'egg fertilisation' by sampling sperm from a female's thelycum post-spawning – this meaning that the resulting egg fertilisation rate is known. Several methods were again examined, focused on both functional-physiological and structural characteristics of the sperm cells.

The calcium ionophore A23187 was trialled for its ability to induce sperm acrosomal reactions (i.e. the physiological process that sperm undergo when they contact eggs, as required to initiate fertilisation) artificially. Existing methods were refined and optimised for *P. monodon* sperm. Thelycal-matured sperm (T-sperm) activation was assessed after 5 min and 30 min exposure to various concentrations of A23187 within its recommended range and compared to activation levels induced by natural egg water, which is known to induce sperm acrosomal reactions.

The Comet assay was used to quantify levels of DNA damage in sperm removed immediately post-spawning from the thelycum of females mated with either wild or

domesticated G8 males. This allowed DNA damage levels to be compared directly against egg fertilisation rates.

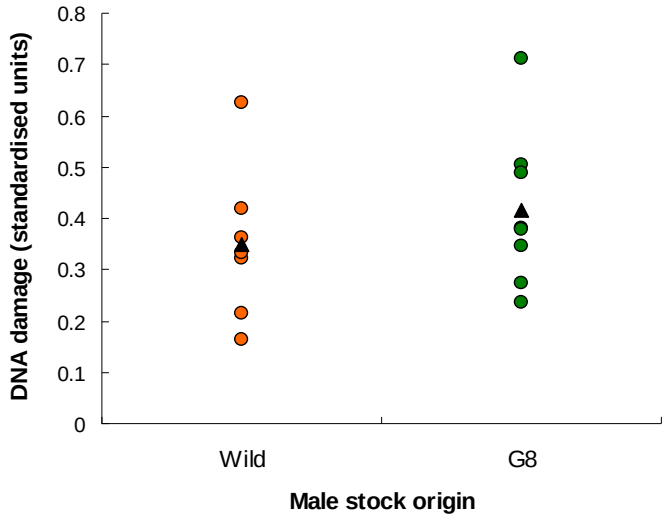
Acrosomal reaction levels of sperm obtained from the thelycum were induced using egg water and compared to egg fertilisation rates. Acrosomal reactions of additional batches of sperm derived from wild and G8 males and some G3 males were also induced to evaluate variability amongst males with differing rearing backgrounds.

Results

When thelycum (T)-sperm were exposed to the artificial acrosomal reaction inducer A23187, less than 5% reacted compared to up to 97.8% reacting when exposed to natural egg water. Considering the high activation capability of the sperm and that the range of ionophore concentrations tested encompassed concentrations known to induce activation responses in sperm from mud crabs and lobsters, it is likely that the Calcium ionophore A23187 is ineffective at inducing an acrosomal reaction in *P. monodon* sperm.

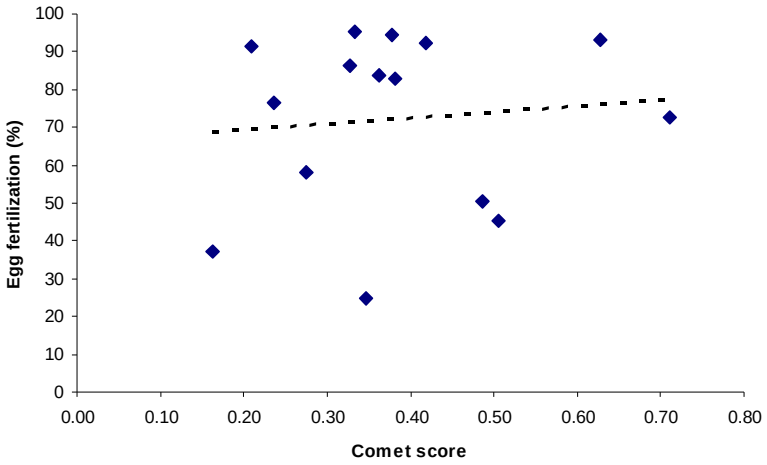
Sperm DNA damage (expressed in standardised units ± S.D.) was slightly higher amongst G8 broodstock (0.42 ± 0.15) than amongst wild broodstock (0.35 ± 0.15) (Figure 4) but the difference was not significant (P>0.05). However, sperm DNA damage levels were low in both stocks with 75% of sperm cells from G8 males and 75.6% of cells from wild males assessed as having no DNA damage. Little variability in DNA damage was evident across all the males examined.

Figure 4.
DNA damage in sperm from individual wild and eighth generation (G8) domesticated broodstock. The ▲ represents the mean DNA damage for each stock. The standardised units shown on the Y-axis describe the mean proportion of cells per each of the visual Comet scores (i.e. ranging from 0-4).



Levels of sperm DNA damage did not correlate with egg fertilisation levels, and certainly there was no suggestion of the negative correlation which would be expected if increasing levels of damage was reducing egg fertilisation (Figure 5). The lack of any correlation was not surprising considering that DNA damage levels were low and egg fertilisation rates above 50% were recorded for most spawns. However, even for the few spawns in which egg fertilisation rates were below 50%, sperm DNA damage was determined to be low, this indicating that it was unlikely to be the main cause of low fertilisation.

Figure 5
DNA damage (as measured by the Comet assay) in sperm taken directly from the female thelycum immediately post spawning and compared against the percent of fertilised eggs.



When sperm acrosomal reactions (ARs) were analysed using only wild females, a strong correlation (R^2=0.65) was found between AR and egg fertilisation (Figure 6). This correlation was weaker (R^2=0.22) when performed using both wild and G8 females; likely resulting from the higher variability in the 'female effect on observed egg fertilisation' introduced by not broadly standardising for female/egg quality. Unlike the majority of the other sperm measures examined, AR scores varied substantially amongst the 30 batches of sperm examined from the wild, G8 and G3 males (Figure 7). Over the AR score range of 0 (no sperm react) to 3.0 (all sperm react), the scores amongst the sperm batches varied wildly from 2.4 (highly reactive sperm) down to 0.2 (mostly non-reactive sperm). Based on the high inter-male variability and the correlation with egg fertilisation, the AR assay using natural egg water does appear to provide a predictive measure of matured T-sperm fertility.

Figure 6.
Correlation between egg fertilisation (%) and acrosomal reaction score of sperm measured from the females thelycum post spawning. The standardised units shown on the X-axis describe the mean proportion of cells per each of the visual AR scores (i.e. ranging from 0-3).

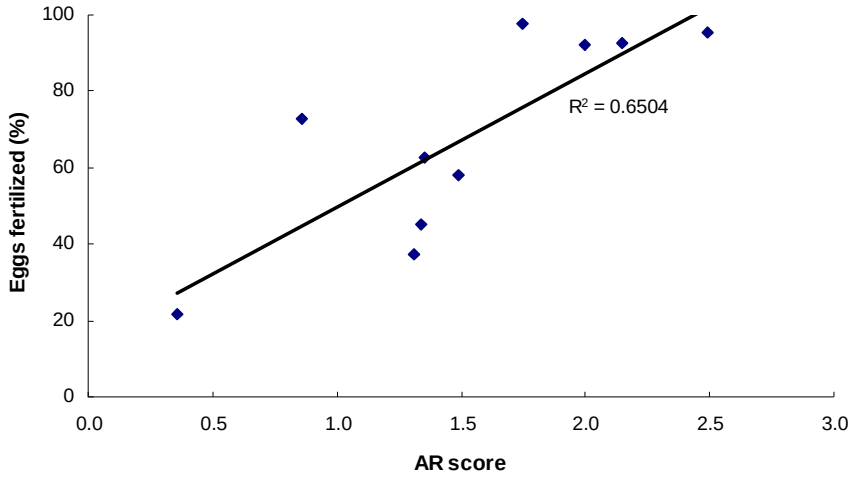
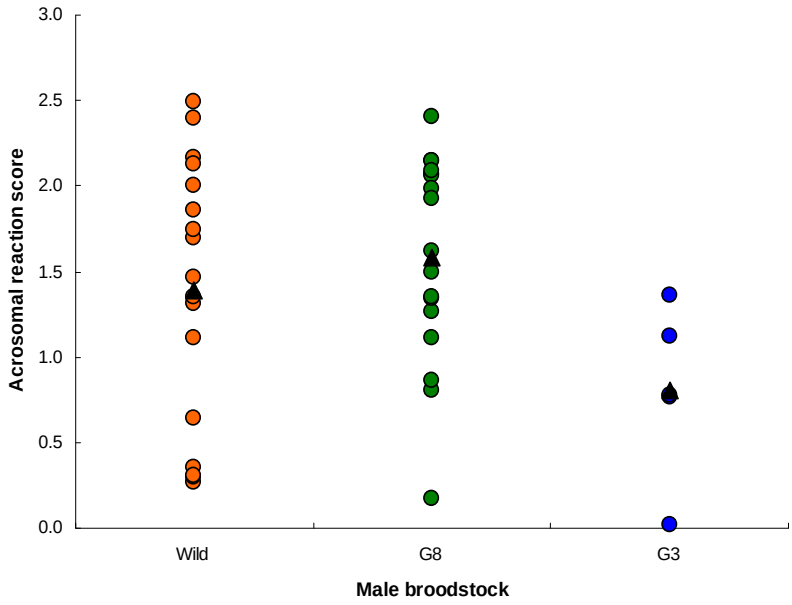


Figure 7.
Acrosomal reaction score of sperm measured from the female thelycum post spawning for wild, eighth generation (G8) and third generation (G3) domesticated *P. monodon* males. The ▲ represents the mean AR score for each stock. The standardised units shown on the Y-axis describe the mean proportion of cells per each of the visual AR scores (i.e. ranging from 0-3).



3.4 Assessing alternative measures of male fertility through histological evaluation of spermatophores from males and the female's thelycum

Methods

Histology was used to examine spermatophores artificially ejaculated from wild, G1, G3 and G8 male broodstock and also spermatophores of wild and G8 males removed from the female thelycum immediately post-spawning.

Results

There were no obvious histological abnormalities or differences in sperm released from 15 normal-looking spermatophores ejaculated from wild, G1 and G8 males. Of the 5 males assessed from each stock, all had sperm normal in morphology (i.e. dark even staining nuclei) except for one wild and one G1 male in which ~25% of sperm had pale staining nuclei.

Histology of abnormal-looking spermatophores ejaculated from G3 males revealed sperm with morphological abnormalities. Sperm from one male was misshaped and degenerated whilst some sperm from two other males had slightly pale nuclei. One spermatophore from another male possessed some 'hollow' sperm devoid of an obvious nucleus, but all sperm from the other spermatophore appeared normal (i.e. dark even staining nuclei). One male also possessed some necrotic debris in one spermatophore while no sperm were found in one spermatophore of another male.

There was no obvious relationship between morphological normality of T-sperm (sperm from spermatophores removed from the female thelycum post-spawning) and egg fertilisation. In 4 of 5 male spermatophores excised from females in which egg fertilisation rates varied widely (i.e. 44%, 84%, 87% and 90%), sperm were scored as normal in morphology. The male spermatophore excised from the other female possessed good dark dense sperm and had a 55% egg fertilisation rate. Of note, it is difficult to draw strong conclusions of these histological assessments of T-sperm morphology and egg fertilisation due to the small number of prawns examined.

Importantly, as spermatophore histology only detected sperm abnormalities in abnormal spermatophores, and typically found normal sperm in normal-looking spermatophores, it seems that histology provided little additional benefit in identifying sperm abnormalities over gross spermatophore observations. Unless future research, using much larger number of animals, can find evidence that sperm abnormalities identified through histology do correlate with observed egg fertilisation rates, it seems that histological evaluations of sperm morphology will not prove useful for predicting egg fertilisation potential of individual males.

3.5 Discussion: To develop an objective measure of 'male fertility'

Correlation between the 'easy measures' and egg fertilisation

The initial experiment, which examined the fertility of sperm derived from different males when impregnated into single females, indicated that none of the male

parameters which can be easily measured, such as spermatophore weight, sperm count, or percent normal sperm, could be linked directly to egg fertilisation; the caveat to this being that this applies in cases where spermatophore morphology and sperm counts/morphology are within ranges not considered atypical or grossly compromised. It appears therefore, that sperm quality characteristics, which aren't captured by these crude observational methods, dictate a male's ability to fertilise an egg. It is likely, however, that in extreme cases where male reproductive tracts, spermatophores and sperm number/morphology are comprised grossly, these easily measured methods could be predictive of poor male fertility. However in such cases gross spermatophore observations will often be sufficient to identify compromised fertility.

Importantly, whether the results of this first experiment were influenced by direct competitive interactions between sperm from different males (when mixed within a female) is not known. However, given that the number of eggs fertilised amongst the spawns analysed varied markedly, irrespective of what males provided spermatophores, and presuming that egg quality did not solely dictate the variability in fertilisation rates, it seems unlikely that inter-sperm competition could have caused all differences in male parental origins of fertilised eggs. It appears far more likely that differences in male parentage of eggs were dictated by the inherent ability of some sperm from one male to fertilise eggs more efficiently or effectively. Furthermore, we can not rule out the possibility that artificial ejaculation of the spermatophores, as required in the experiment, may have caused variability in the maturity (i.e. and so subsequent viability) of the sperm at egg fertilisation. But notably, only spermatophores that were easily extracted and normal in morphology were included in each insemination pairing; and therefore all efforts were made to use only seemingly mature spermatophores.

Measures assessed from S-sperm

Based on the findings of this first experiment, other methods were trialled on sperm from male spermatophores (S-sperm), with the initial aim being to determine whether the sperm quality characteristics of each method differed between males. These methods focused on different aspects of sperm cell structure, chemistry and physiology, but none were able to discriminate between males to a degree necessary to consider optimizing it further. The method that provided some degree of male fertility discrimination was the Comet assay. However, whilst showing some promise, crude visual assessments of general sperm morphology were generally found to provide comparable discriminatory power. Hence, based on the present findings, and due to its relative complexity, we did not find compelling evidence that the Comet assay would provide the level of discrimination that would justify its user for most current commercial applications. However, given the method does have an ability to discriminate based on certain sperm characteristics, it may be that the method could be used, possibly as one of several measures, in alternative applications of assessing sperm quality.

Measures assessed from T-sperm

With limited success in finding reliable measures of male fertility based on sperm taken directly from the male spermatophore, methods were trialled using sperm that

had been capacitated after a period within the female thelycum (T-sperm) following spermatophore insemination. These T-sperm evaluations aimed to determine whether the sperm quality characteristics of each method were correlated with egg fertilisation rates following spawning. Of the methods trialled, only the acrosomal activation assay using natural egg water (i.e. EW-AR assay) showed potential. The EW-AR assay produced high levels of activation in sperm, and detected clear differences in sperm reactivity between males. Concomitant with this, the levels of sperm acrosomal reactivity induced by natural egg water, showed an ability of this assay to predict egg fertilisation rates. For example, when standardised broadly for egg quality by only evaluating fertilisation rates of eggs spawned from wild females (presumed, and found, to be more uniform) a significant (moderate) correlation was found. Given the likely 'noise' due to the effects of 'inter-female variability in egg quality' on observed fertilisation rates, the presence of moderate correlation between egg water-acrosomal reactivity of T-sperm and egg fertilisation rates suggests this assay provides a useful predictive measure of male fertility, providing it can be applied practically.

Developing a practical method

From an on-farm implementation context, methods that assess the potential fertility of S-sperm derived from male spermatophores have clear advantages over methods that assess the potential fertility of T-sperm derived from the female thelycum. As spermatophores can be collected readily in the hatchery, and at least for certain parameters, the S-sperm can be assessed readily in the hatchery, these tissues provide the preferable sample source for commercial evaluation of male fertility. However, as no characteristics of spermatophores or S-sperm were found to have the discriminatory power to distinguish between individual males, it seems that currently S-sperm assays can provide only a broad means for gauging male fertility (i.e. providing a means to identify poor males, but not to distinguish between average or highly fertile males).

Of all measures trialled, only a single measure performed using T-sperm was found to have significant potential to assess male fertility; this being the egg water-acrosomal reactivity (EW-AR) assay.

Whilst the EW-AR assay using T-sperm does provide a means of assessing male fertility, employing this technique in a commercial setting would be challenging, and for many applications, it may be prohibitive. Firstly, the assay does require invasive sampling of an impregnated female to collect sperm; which can either be through thelycal biopsy or by sacrificing the female. Certainly, such invasive sampling is not conducive if having to be performed on highly valuable female broodstock. Secondly, such assays using T-sperm are time consuming as they require a period of at least 3 days beyond female insemination with spermatophores from 'relevant' males before the assay can be performed. Thirdly, the EW-AR assay requires egg water to be collected and stored appropriately. While not difficult or expensive to do, this does add another level of complexity when performing the assay. Finally, the assay requires a degree of 'technical capability' over many other assays, and takes 15-30 minutes to complete; thus making it challenging to apply on a large-scale (i.e. to assess many males).

To implement the EW-AR assay commercially for the purpose of assessing the relative quality of individual males before they are used for mating would certainly seem, at the very least, a significant challenge. For this purpose, 'surrogate' females would need to be artificially inseminated (i.e. which requires tracking moulting patterns of these females) with spermatophores from the 'putative' male broodstock. These females would then need to be invasively sampled to obtain T-sperm, following the subsequent 3 day period of sperm capacitation, and the EW-AR assay performed. To do this at large scale, for the purposes of identifying fertile males at the 'individual' level, would certainly appear impractical for most Australian hatcheries. However, there is likely more potential to employ the assay commercially for the purposes of monitoring 'cohorts' of males. In such cases, the assay could be performed using a small subset of females, which are chosen from the population for invasive/sacrificial evaluation and which have previously mated by males from the 'relevant' cohort. Such evaluations could allow long-term monitoring of a large population (e.g. sacrifice of a few females at regular intervals from a pond population for extraction of T-sperm) or more immediate evaluations of males prior to there use in the hatchery (e.g. placement of 'surrogate' females along with males from the relevant cohort for natural mating - and subsequent T-sperm extraction and assessment).

The confirmation that the EW-AR assay is associated with egg fertilisation does provide a basis for guiding future research to identify a practical measure of S-sperm virility, which should focus on the characteristics of S-sperm that dictate its high acrosomal reactivity potential once matured within the female thelycum. Based on knowledge gained through this project, the sperm characteristics involved are not likely to be directed by membrane integrity or mitochondrial function, and in all likelihood could be multi-factorial in nature. In this eventuality, development of a single fertility assay for male *P. monodon* based on qualitative or quantitative measurement of a single physiological, chemical or structural sperm cell parameter appears somewhat presumptuous.

Based on our current findings, there is still no easy means to reliably rank the relative 'fertility' of individual males 'tank-side' in the commercial hatchery. However, the protocols developed, and the ranges of parameters reported in the attached industry document (Appendix 2), provide a refinement in guiding 'tank-side' evaluations of whether a male is within the normal range; and thus can aid in the process of weeding out males of poor fertility. Furthermore, through the EW-AR assay applied to T-sperm, there is a means for more reliable evaluations of male fertility, which may have commercial application for monitoring fertility of cohorts of males. To implement this assay requires only a modest investment in equipment to collect and store egg water, and a degree of technical training to undertake the assay. If more amenable methods of evaluating fertility through use of S-sperm remain a priority, research would need to focus on the range of characteristics of the sperm that predetermine its ability to capacitate in the female thelycum.

4. To undertake histology to identify the prevalence of sperm morphology abnormalities amongst wild-caught males and domesticated males reared under different environmental conditions

Rational & methods

Histology of cephalothoraxes of experimental prawns reared in ponds and tanks throughout the project was performed to assess male RT&SD development as well as the general health of both males and females. Of note, these analyses found no gross morphological defects in male RT&SD at any sampling point for prawns reared in any ponds or tank treatments under various conditions. However, when sperm cells in different tract sections were examined closely, morphological abnormalities, which had not been described previously, were evident in many prawns. As a consequence of the discovery of these uncharacterised sperm abnormalities, a concerted effort was made, particularly in Year 2, to examine for their presence in different pond and tank groups. Notably, these examinations found no consistent patterns in the occurrence of the sperm abnormalities in males from any of the pond or tank treatments, but rather these abnormalities were found dispersed across the range of experimental treatments.

Due to the relative absence of gross morphological abnormalities observed in the reproductive tract/spermatophores, and as sperm abnormalities did not specifically associate with any experimental treatments, evidence of histological abnormalities were subsequently examined using sperm collected from males from much broader 'origin' groupings; as noted below:

- Prawns being reared commercially on Queensland farms [shore-based]
- Wild broodstock from the east coast (EC) of Queensland sourced from hatcheries [off-shore]
- Wild broodstock sourced from the Northern Territory (NT) sourced from hatcheries [off-shore]

Results

Based on the first observations of these abnormal sperm cells, the syndrome was initially coined as 'hollow sperm syndrome (HSS)'; in reference to an apparent abnormality where the sperm appeared 'hollow (H)' and devoid of nuclear material as viewed through histology, (H, Figure 9, for comparison with normal sperm, see

Figure 8). Subsequent observations found other sperm abnormalities were also present, such as sperm displaying pale-enlarged (PE, Figure 10) nuclei that stained poorly and contained centrally located chromatin. Subsequent transmission electron microscopy (TEM) showed 'electron-dense' chromatin in the nucleus of H-sperm to be reduced markedly. More chromatin was visible in PE-sperm but it was either marginated at the nuclear membrane or dispersed in an unorganised manner throughout the nucleus. The term HSS is used from hereon in reference to all observations of sperm abnormalities, both hollow; pale-enlarged; but also several

other abnormalities of the sperm cells which were subsequently described. However our observations continue in order to understand the nature of these abnormalities and current indications are that the less extreme abnormality, PE sperm, may represent a normal phase of sperm DNA "decondensation."

Histology found evidence of these morphological abnormalities in sperm of males from all these broader groupings. Averaged across all males for which reproductive tracts were examined by histology, 79% possessed detectable levels of H-sperm in the testes (at an estimated average severity was 31%), and 20% and 13% possessed detectable levels of H-sperm in the vas deferens and spermatophores, respectively.

No association between the incidence and/or severity of HSS was apparent between the seasonal period in which the prawns were collected; viral infection loads; or nutritional condition of the prawns. The only possible association apparent seemed to be a link between HSS and the 'rearing location or source' of the prawns. Higher incidences of sperm abnormalities in the testes and vas deferens were evident amongst males examined from shore-based locations (i.e. BIRC ponds, CSIRO tanks, commercial farm ponds in Southeast Queensland) than amongst off-shore locations (i.e. wild male broodstock from EC and NT) (Figure 11). Amongst the shore-based prawns, testicular H-sperm and PE-sperm were detected in 14-59% and of 21-52% males, compared to rates of 0-16% and 0-24% of off-shore males, respectively. Similarly, rates of vas deferens H-sperm and PE-sperm were higher in shore-based males (4-18% and 0-10%) compared to off-shore males (0-4% and 0%, respectively).

Discussion

As noted earlier, observation under the light microscope of sperm cells, sampled from spermatophores collected from males reared across the range of different experimental pond and tank treatments, found few deviations from the expected morphology of sperm. However, when male cephalothorax tissue sections were examined by histology, a range of sperm 'anomalies', previously unreported, were observed. These anomalies (i.e. abnormalities) were termed broadly as hollow sperm syndrome (HSS) initially as sperm appeared to be devoid of typical nuclear material, and subsequently a range of more subtle sperm abnormalities were identified.

Figure 8
P.monodon testes H&E x1000: Normal sperm.

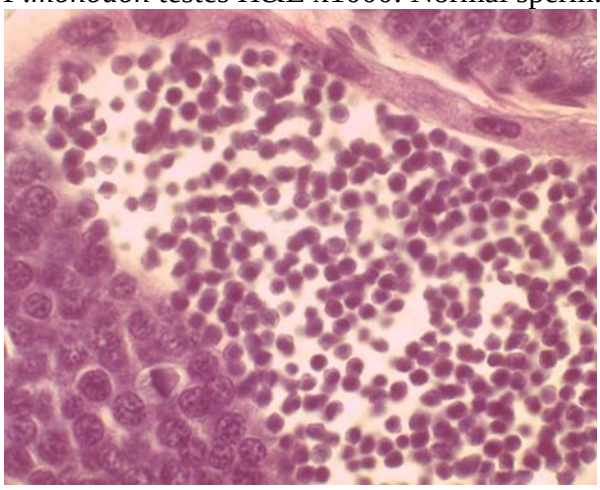


Figure 9
P.monodon testes H&E x1000: Presence of hollow sperm cells with chromatin margination.

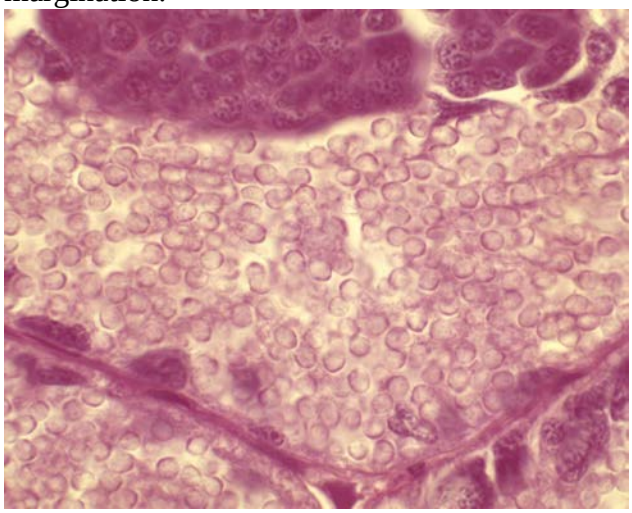


Figure 10
P.monodon testes H&E x1000: PE sperm (P) displaying reduced staining and increased size relative to normal sperm (N) in the testicular lobule.

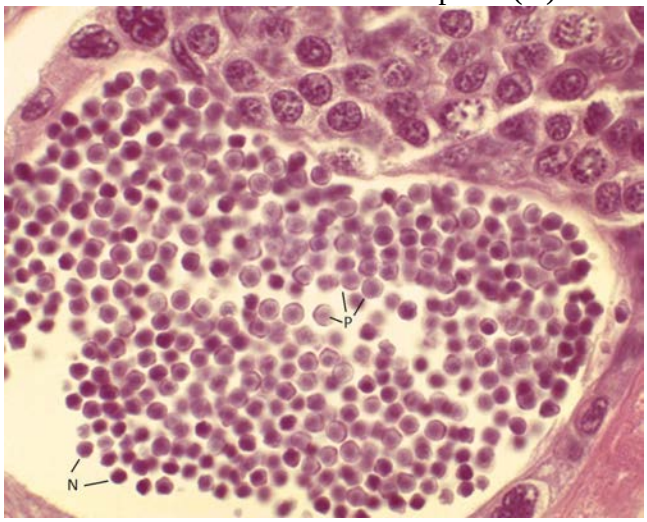
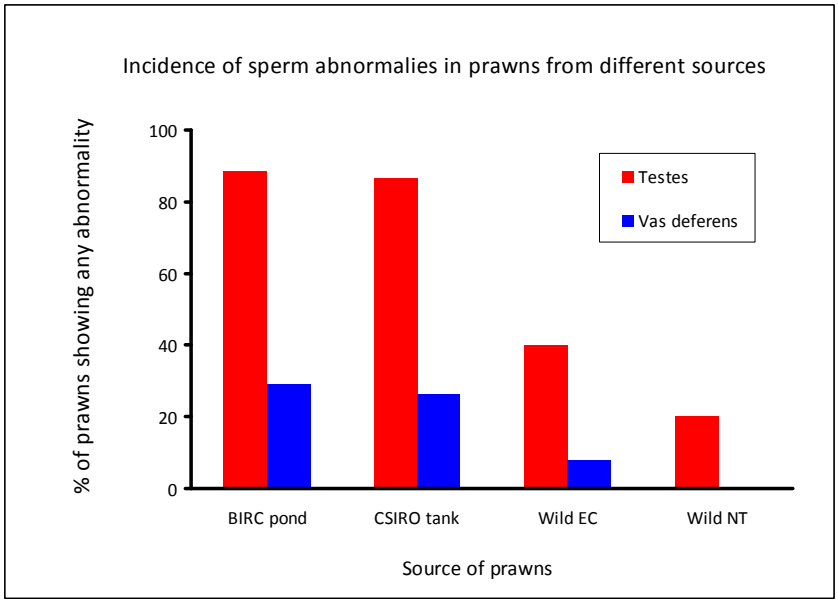


Figure 11.
Percentage of male prawns sampled from different origins showing any signs of sperm abnormalities in their testes and vas deferens as determined through histological evaluation.



Broad general patterns in these sperm abnormalities gradually emerged. Hollow sperm (HS) occurred more frequently in the upper tract regions such as the testes and declined progressively in frequency amongst sperm positioned further down the vas deferens and in fully-developed spermatophores. Initially it was not clear as to whether HSS might be a normal biological process associated with some mechanism for preventing defective sperm from being packaged within spermatophores. However, as more prawns were examined it became apparent that these abnormal sperm could enter the spermatophore. As this phenomenon has not been reported previously, it appeared to be atypical. Indeed, histology on archived blocks found no evidence of hollow sperm occurring commonly in wild-caught males or wild males that had spent only brief periods in captivity. In contrast, its incidence was higher in wild males that had been reared for considerable times in captivity and amongst domesticated males that had spent all their life in captive rearing systems. However, its severity varied substantially amongst individual males from any particular group/cohort and amongst different cohorts, and it was not seen ubiquitously in all males.

The HSS abnormality was not an artefact due to poor or uneven tissue fixation for histology. It was also associated more commonly with stocks reared near shore and in captive land-based culture systems and not associated strongly to any of the 'experimental' stressors examined in this project. However, importantly, we have yet to clarify and quantify any impacts of HSS on overall male fertility. Amongst limited numbers of prawns mated experimentally in this project to assess egg fertilisation rates, no consistent negative impacts of sperm abnormalities were observed. However, numbers of abnormal sperm detected amongst males from these cohorts were

relatively low; and the impacts that higher levels of sperm abnormalities would have on egg fertilisation rates are as yet unknown.

A problem expressed as unusual sperm morphology, or even normal morphology present in a part of duct where it shouldn't persist, could be explained by an abnormal slowing in sperm development rate as the cells move down the duct. There is a possibility that the PE sperm found here reflect a normal phase of sperm development. Some dispersion of chromatin is expected during re-organisation in the sperm cell during formation, but there is so far no precedent at all in the literature for sperm that appear to lack stainable DNA being present in the vas deferens.

Further investigations of the occurrence and development process of HSS over time amongst males from different sources and reared in differing environments are needed to understand how it develops and understand its cause. Additional examinations of egg fertilisation from females inseminated with spermatophores containing abnormal sperm are also needed to conclusively establish the impact of HSS on fertilisation rates. If an association is found, histology on the reproductive tracts of males could provide one diagnostic indicator of poor male fertility in the hatchery.

Whilst speculative, the nature and presentation of HSS suggests that it might arise in response to males being exposed to agricultural and/or urban chemical residues that enter aquatic environments and which would be expected to be more concentrated in estuaries and near shore environments and in regions proximal to farming and urban activities. Due to the abnormalities being seen in reproductive systems of males, of particular potential interest might be chemicals that can function as endocrine disruptors.

5. General Discussion

Rearing broodstock in ponds

The inherently lower costs of constructing and managing ponds, as compared to smaller-scale tank and raceway systems, makes ponds an attractive option for rearing *P. monodon* broodstock. Certainly, including pond-rearing as a part of the broodstock cycle would be the most effective means to up-scale production of domesticated broodstock. Of course, the prerequisite for including pond-rearing within the cycle is that these systems can reliably produce healthy and highly fecund broodstock.

The current project found that male broodstock reared in ponds over two successive seasons showed no signs of compromised development of the reproductive tract or spermatophores. Furthermore, when males were subjected to a range of extreme/sub-optimal environmental conditions in pond and tank experiments, no evidence was found for compromised development of the reproductive tract or spermatophores. Clearly, within the scope of the parameters tested, pond-rearing at low densities (less than 8 m^{-2}) does not appear to pose an inherent risk for the production of male *P. monodon* broodstock. The compromised development of the reproductive tract and spermatophores of pond-reared males observed in former projects seems most likely to have been caused by either transient agents and/or unidentified factors that the stocks were clearly not exposed to in present study.

Certainly none of the trialled environmental or dietary 'stressors' applied in the current experiments seem to pose significant risk, at least in isolation, to gross reproductive development of males.

Despite the absence of abnormalities in development of the tract and spermatophores, morphological anomalies in sperm (H-sperm) were identified at varying prevalence across the experimental stocks. However, as yet there is no evidence linking these sperm anomalies to egg fertilisation, and so we can not as yet presume that these anomalies are significantly impacting on male fertility. Only future research focused on examining the development of these anomalies, and identifying their impact on egg fertilisation, can determine the role that these anomalies are playing in male fertility.

Importantly, insurance against issues of reproductive development of broodstock is best provided by a combination of multiple location rearing and long-term monitoring of the stocks. If using ponds for large-scale broodstock production, it would still seem prudent to rear subsets of core stocks in at least one other location. This alternative location would ideally be isolated both in terms of the physical site and the influent water source, but also in terms of system operations (e.g. on a different electrical circuit). While in future other options may also become available to commercial operators (e.g. gamete preservation), currently it would seem that multiple location rearing would provide the best insurance. Sacrificing small numbers of males to enable evaluation of gross reproductive development and health of the males at regular intervals (e.g. 3, 6, 9 months of age) in the different systems is also important to provide warning of compromised development; and therefore for influencing decisions when selecting stocks to use as future broodstock.

Measuring male fertility

The current project found that the simplest measures of male fertility that could be performed 'tank-side' in the hatchery do not provide a means to discriminate 'fertility' between individual males. Measures such as spermatophore morphology; spermatophore weight; and S-sperm number provide a useful means to weed out males of very poor fertility. However, these measures do not represent the 'quality' characteristics that influence egg fertilisation, and so within normal bounds, they do not provide a means to predict fertility of individual males.

The EW-AR assay performed using T-sperm does seem to provide a reliable means to estimate male fertility, but due to both the requirements of obtaining the T-sperm sample and undertaking the assay, this measure is much more challenging to use for many commercial applications. The EW-AR assay likely has some potential to evaluate 'cohorts' of males; by collecting T-sperm from 'surrogate' females which have previously mated naturally with males from the relevant cohort. However, certainly the EW-AR assay would be challenging to use for predicting the fertility of individual males at a large scale, as this would require artificial insemination of large numbers of females, even before the assay is performed. Given these requirements of the EW-AR assay, it does seem likely that its main application to the Australian industry would be to evaluate cohorts of males prior to their introduction into the mating tanks prior to commencement of the hatchery run.

The confirmation that the EW-AR assay is associated with egg fertilisation provides a basis to guide any future research efforts to develop a more 'practical' measure of male fertility; which would necessarily focus on the characteristics of S-sperm that determine its high acrosomal reactivity potential once matured within the female thelycum. However, based on current findings and evidence from other species, it is presumptuous to expect that a *P. monodon* male fertility assay, based solely on qualitative or quantitative measurement of a single physiological, chemical or structural sperm cell parameter, will be found.

Options for future broodstock production and management

Results from the present study do suggest ponds can play an important role in large scale broodstock production. Such large-scale rearing systems provide both the opportunity to produce large numbers of broodstock for commercial use, but also to increase intensities of selection in genetic improvement programs. Importantly, ponds carry an inherently higher risk of environmental variation than smaller (often enclosed) systems, due to their typically higher exposure to climatic and influent water-quality variability, and due to the more variable nature of green-water systems. Consequently, consideration has to be given to the risks of such variability when deciding on the period of the rearing cycle for which ponds are to be used at the specific rearing location. For example, companies need to weigh up the risks and benefits of using ponds for summer rearing only (e.g. for ages 1 to 6 months) versus for longer durations (e.g. for ages 1 to 10 months). The approaches to management also need to be considered. Certainly, companies both in Australia and overseas are now operating *P. monodon* breeding programs using low-density pond-rearing for a significant portion of the rearing cycle; and no systematic problems of reproductive development (of either sex) in these pond stocks has been reported.

Notably, most of the ponds used in the present project were covered, providing a degree of climatic buffering. If companies are solely using open ponds, the risks of heightened variability in the pond environment are much greater due to climatic variation. Similarly, given the low-densities used in broodstock ponds, rates of water-exchange can be quite low reducing risks of introducing undesirable contaminants or agents into the systems via the influent water. However, the risks associated with the introduction of any required quantities of water into the ponds throughout the rearing cycle, and the scope for water treatment at the volumes required, need to be considered when deciding on the role pond-rearing will play in a companies broodstock production strategy.

Despite the potential of ponds as a broodstock production system, there are still advantages of using smaller scale rearing systems in terms of environmental control and to allow isolation of different cohorts/groups of stocks. Such smaller scale systems, used to house replicate stocks, can also provide invaluable insurance for pond systems. But certainly, even these smaller scale systems do not remove all risk of broodstock production. Importantly, monitoring of the reproductive development and health of stocks in these smaller scale systems is as important as in the larger pond systems; and given the smaller number of animals able to be maintained, companies certainly need to plan strategies which provide scope to allow sacrifice/monitoring of the stocks in these systems.

Beyond rearing fecund broodstock in the broodstock production systems, the commercial operators also need to effectively manage the broodstock selected for use in the hatchery (i.e. both for commercial purposes; but also for reproducing the breeding lines). As mentioned above, while highly accurate predictive measures of male fertility are not amenable for hatchery use, there are means by which commercial operators can screen male broodstock for 'fertility' prior to, or at the point of entry, into the hatchery maturation systems. Importantly, as most Australian hatcheries and breeding programs rely on natural mating, it is important that mating success is high post-transfer of the stocks into the maturation systems. Approaches to improving likelihood of mating success (e.g. heightening sex ratios towards males) and monitoring mating success (potentially through the use of 'surrogate' females co-stocked for this purpose at the initial introduction of the males into the maturation systems) can provide confidence that mating is adequate, prior to the ablation and spawning of the valuable female broodstock. Artificial insemination (AI) could also play a role in ensuring valuable females are mated, particularly for reproduction of core genetic lines (and to make specific genetic crosses). However, the added labour and husbandry requirements of AI preclude this procedure for many Australian operators in most instances.

Through a combination of monitoring fertility of males in the broodstock rearing system (prior to introduction to the hatchery); crude screening of individual males being introduced into the hatchery; and monitoring of mating immediately after introduction of the males into the hatchery, commercial operators provide their best chance to heighten fertilisation and nauplii production from their hatchery broodstock.

Future research

The project identified the following topics of greatest value for future and on-going research and data collection.

- Determining the characteristics of S-sperm that predict the acrosomal reactivity of the sperm post maturation within the thelycum
- Collating of information on environmental conditions and prior rearing history of (any future) cohorts of males found to have significantly compromised reproductive development
- Establishing the impact of HSS on male fertility; and investigating the causes of the syndrome, particularly amongst domesticated males
- Examining fertility in younger males selected directly from the commercial pond environments to determine whether this could provide a future strategy for intense commercial pond selection

6. Benefits and Adoption

The benefits of this project are to identify approaches to improve the reproductive outputs of domesticated *P. monodon* broodstock; by identifying appropriate rearing systems and providing methods to improve hatchery operations. The results found ponds provide a reliable means in which to rear male broodstock; and certainly provide a cost-effective option for large-scale broodstock production. Experiments

demonstrated that male fertility does significantly vary between males, and can impact significantly on hatchery output. One trialled measure to assess male fertility (EW-AR assay of thelycal-capacitated sperm - T-sperm) was confirmed to be associated with egg fertilisation; thus providing a predictive measure of fertility. Approaches to evaluating male fertility have been proposed to assist in improving hatchery production. Furthermore, an industry document (Appendix 2) is provided to assist in the identification of likely causes of the fertility problems in the hatchery, and to review the practicality and reliability of various measures of male fertility. Typical 'normal' ranges of male reproductive parameters, these being the prerequisites for male fertility, are summarised in the document.

Pond-rearing has already been adopted as a means for rearing domesticated stocks on commercial farms; and no gross problems of male reproductive development have been reported in these pond-reared males. The current project results and the successful adoption on-farm should give confidence to other industry members of the viability of using low-density pond-rearing for *P. monodon* broodstock production. A document prepared for industry-use (Appendix 2) has been developed alongside this project. This document focuses on identifying causes of low fertility (i.e. and more broadly low nauplii output) and outlines methods for evaluating male fertility; based on best current knowledge. The information within the document will be supported through direct contact between interested industry members and CSIRO/DEEDI staff. If there is sufficient interest, CSIRO staff can assist interested industry members in the protocols to perform the EW-AR assay.

7. Further Development

Based on the results provided, industry members considering developing their own domesticated lines and establishing breeding programs should consider the benefits/risks of pond-rearing. Certainly, in many instances, inclusion of a pond-rearing phase as part of the broodstock production system would seem warranted and could significantly reduce costs of broodstock production.

Approaches to monitoring male reproductive development and mating have been proposed. Industry members interested in adopting any approaches can contact CSIRO/DEEDI staff for more information, or to discuss the options in more detail.

If the industry feels that continued efforts needs to focus on the development of an easier predictive assay than was able to be achieved in the present study, then further research would need to focus on determining the characteristics of S-sperm that predict the acrosomal reactivity of the sperm post maturation within the thelycum.

In terms of other research and on-going data collection, the most logical extension of the project would be to monitor RT&SD in commercial stocks over coming seasons. Coupled with this monitoring, more comprehensive investigation of HSS in the domesticated stocks, and determining the impact of this syndrome on egg fertilisation, would be critical to know whether this syndrome is of commercial relevance.

Certainly, the reproductive performance of domesticated males typically remains below wild-caught males (CSIRO unpublished). However, it is the overall

profitability of seedstock supply and pond grow-out performance enabled by the implementation of a breeding program that needs to be considered when assessing the performance of the broodstock; rather than comparisons of reproductive outputs from wild-caught versus domesticated broodstock. The priority given to further research to improving male broodstock performance should thus depend on the target reproductive output required to maintain overall profitability (and practicality) in the breeding programs. Commercial operators will therefore have to assess the economic value of improving male performance, versus other contributing aspects of the breeding program, when determining the need for ongoing research toward improving male fertility.

8. Planned Outcomes

- *A practical regime for long term monitoring of male reproductive development in commercial broodstock system.*

Approaches to long term monitoring of male reproductive development have been proposed. Through sacrificial examination of the reproductive tract of small numbers of males at regular time points (e.g. 3, 6 and 9 months); through cohort evaluations of spermatophores/sperm of males prior to introduction into the hatchery; and through crude assessments of male spermatophores/sperm and mating success in the hatchery, commercial operators can provide the best opportunity to increase male fertility in the hatchery and improve seedstock (nauplii) output.

- *A best-practice summary of cost-effective commercial scale production of domesticated monodon broodstock based upon the scope of the findings of the project*

Rearing of broodstock at low-density in ponds produced males showing normal reproductive development in two successive seasons. Practices adopted in managing these ponds were largely typical of standard pond rearing practices. Given these findings, and the obvious cost-effectiveness of pond systems compared with smaller rearing systems, best-practice recommendations for broodstock production would be to follow existing practices of broodstock management; but with rearing stocks at densities below 8 m^{-2} and feeding stocks on a high quality (high protein) pellet.

The other major recommendations noted are in relation to heightening monitoring of the male stocks; incorporating the use of the different measures of male fertility; and the rearing of stocks at multiple sites for insurance.

9. Conclusion

1. Male fertility was found to have significant effect on observed rates of egg fertilisation
2. Ponds operated according to current industry practices, with minor changes to stocking densities and diet, present a cost-effective option for up-scaling production of domesticated male broodstock
3. Relatively simple measures of male fertility are described to monitor male maturation in the commercial environment; however these can not reliably discriminate the relative egg fertilising capability of individual males
4. A more involved assay, the egg-water acrosome reaction (EW-AR assay) was shown to be linked to egg fertilisation; this assay has potential to be applied commercially for evaluation at the 'cohort' level, but less likely to have application to discriminate between individual males
5. Further understanding of the egg-water acrosome mechanism's link to fertilisation may open the way to develop a reliable and more commercially manageable measure of sperm potency directly using sperm from males (S-sperm).

10. References

Burke, M., Coman, G., M., Kenway, Macbeth, M., Cowley, J., Knibb, W. (2008) "Practical, feasible and low cost genetic selection of *P. monodon* for increased profitability." Final milestone report for the Fisheries Research Development Corporation (FRDC) project 2005/205.

Coman, G.J., Arnold, S. J., Jones, M. J., Preston N. P. (2007). Effect of rearing density during maturation on growth, survival and reproductive performance of domesticated *Penaeus monodon*. Aquaculture 264, 175-183.

Coman, G.J., Arnold, S.J., Peixoto, S., Crocos, P.J., Coman, F.E., Preston, N.P. (2006). Reproductive performance of reciprocally crossed wild-caught and tank-reared *Penaeus monodon* broodstock. Aquaculture 252: 372-384.

Coman, G.J., Crocos, P.J., Arnold, S.A., Keys, S.J., Preston, N.P. (2005). Growth, survival and reproductive performance of domesticated Australian stocks of the Giant Tiger Prawn, *Penaeus monodon*, reared in tanks and raceways. Journal of the World Aquaculture Society 36: 464-479.

Coman, G., Kenway, M., Cowley, J., Burke, M. (2008) "Genetic Improvement of *P. monodon* – establishing commercial readiness." Final milestone report for the Fisheries Research Development Corporation (FRDC) project 2006/205.

Kenway, M. 2000. Development of *P. monodon* selective breeding at AIMS. Australian Prawn Farmers Association conference 2000, Townsville.

Pratoomchat, B., Piyatiratitivorakul, S., Menasveta, P., 1993. Sperm quality of pond-reared and wild-caught *Penaeus monodon* in Thailand. Journal of the World Aquaculture Society 24, 530-541.

Preston, N., Burke, M., Coman, G., Cowley, J., Kenway, M., Knibb, W., MacBeth, M., Young, N., Wilson, K. (2007). “Understanding and removing the barriers to *Penaeus monodon* domestication.” Final milestone report for the Fisheries Research Development Corporation (FRDC) project 2002/209.

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12. List of Appendices

Appendix 1: Additional information

Increasing seedstock production of domesticated giant tiger prawns (*Penaeus monodon*)

Project No. 2008/756

APPENDIX 1 – ADDITIONAL INFORMATION

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

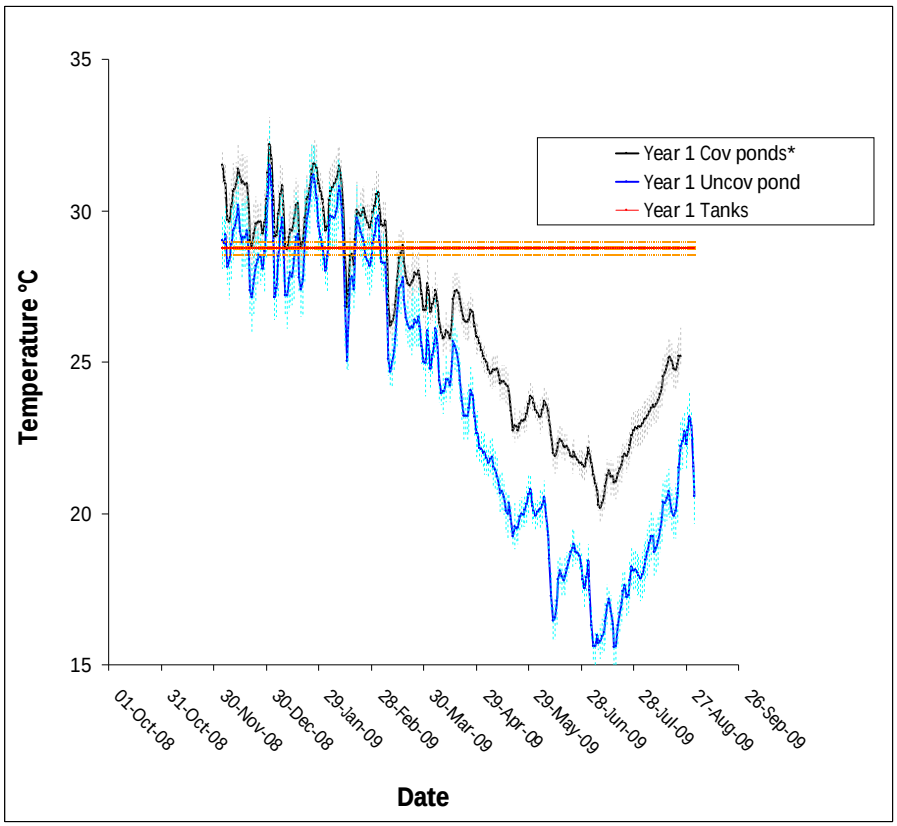
The Australian Seafood CRC is established and supported under the Australian Government's Cooperative Research Centres Programme. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.

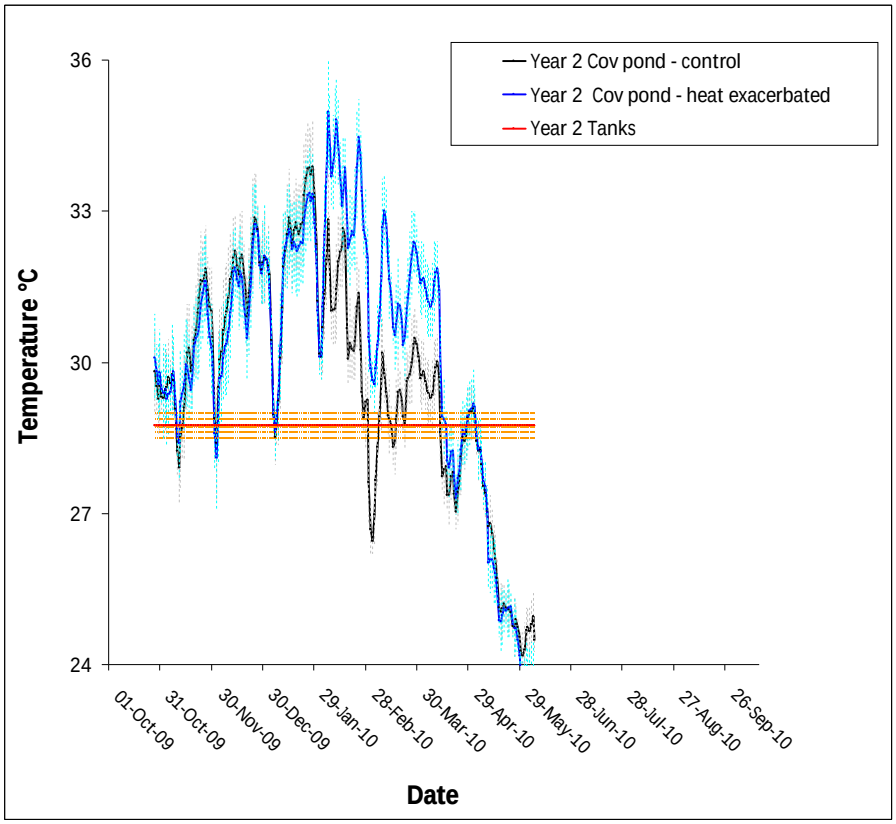
Australian Government
Fisheries Research and Development Corporation

CRC AUSTRALIA

Appendix 1.1

Figure of Mean (Max. and Min.) temperature profiles throughout the pond grow-out period in the BIRC ponds and CSIRO tanks in the two years of the study. Year 1 Cov ponds* represents mean values for the three covered ponds (2, 4, 8m^{-2}).





Appendix 1.2

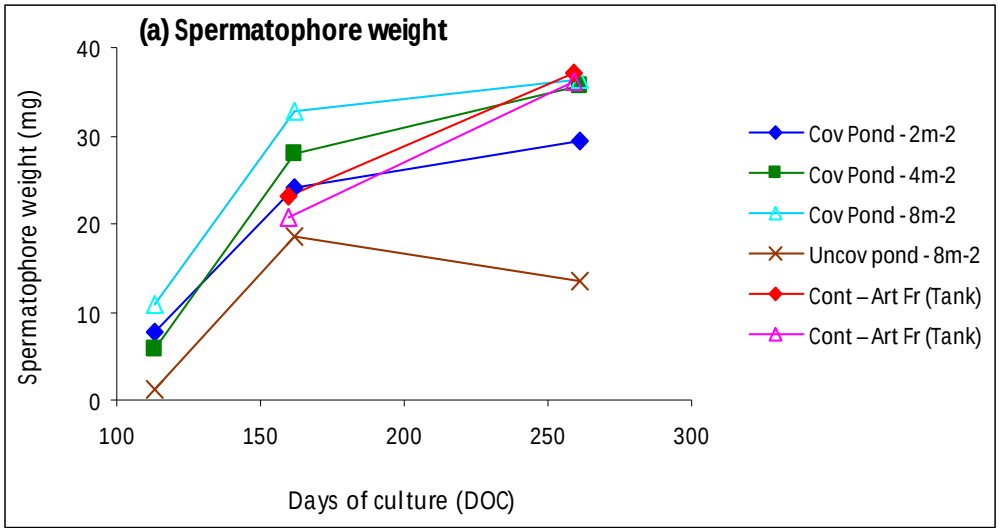
Summary table of treatments examined in the pond-treatment comparisons and pond versus tank comparisons undertaken over the 2 years of the study.

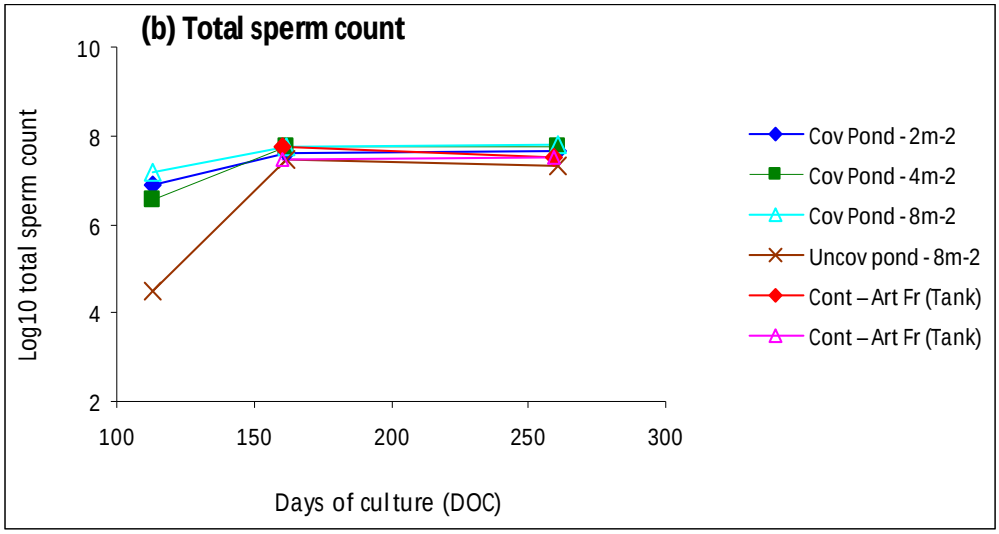
Year	Pond or tank type	Temperature treatment	Density treatment [1]	No. of ponds used [2]	Treatment figure code [3]
1	Covered pond	Pond-managed to retain heat during winter	2 m^{-2}	1	Cov Pond - 2m^{-2}
			4 m^{-2}	1	Cov Pond - 4m^{-2}
			8 m^{-2}	1	Cov Pond - 8m^{-2}
	Uncovered pond	Pond subjected to ambient heat loss in winter	8 m^{-2}	1	Uncov Pond - 8m^{-2}
	Covered tank	Temperature controlled	Standard management [4]	na	Tank - control
2	Covered pond	Pond-managed to avoid excessive heat in summer	2 m^{-2}	1	Cov Pond - control
		Pond-managed to exacerbate heat in summer	2 m^{-2}	1	Cov Pond – heat exacerbated
	Covered tank	Temperature controlled	Standard management [4]	na	Tank - control

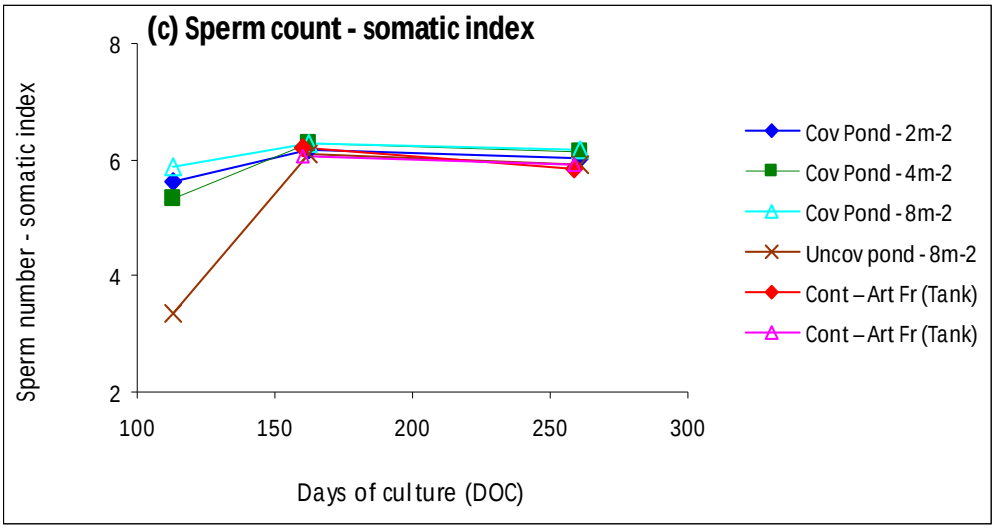
[1] Densities cited for ponds are the densities at stocking
[2] Minimum of 3 tanks used
[3] Codes used relate to treatment presented in Appendix 1.1
[4] Tanks are managed by a progressive protocol of thinning over time

Appendix 1.3

Figure of (a) spermatophore weight, (b) total sperm count, and (c) sperm count-somatic index for male *Penaeus monodon* over time in the different pond and tank treatments in year 1 trials.







Appendix 1.4

Summary table of treatments examined in tank-treatment comparisons undertaken over the 2 years of the study.

Year	Holding Tank system	Stress tank system [1]	Temperature treatment [2] (Max./Min.)	Salinity treatment [3] (Max./Min.)	Diet treatment	Treatment figure code [4]
1	10-t & Flow-through	Unhandled (10-t & Flow-through)	Control temperature (29.0°C / 28.5°C)	Control salinity (36‰ / 35‰)	Artificial & fresh-frozen	Cont -Art Fr
					Artificial	Cont -Art
		Handled (5-t & Low-flow)	Control temperature (29.0°C / 28.5°C)	Control salinity (36‰ / 35‰)	Artificial & fresh-frozen	Cont temp-Art Fr
					Artificial	Cont temp-Art
		Handled (5-t & Low-flow)	Low temperature (29.0°C / 19.0°C)	Control salinity (36‰ / 35‰)	Artificial & fresh-frozen	Low temp-Art Fr
					Artificial	Low temp-Art
2	10-t & Flow-through	Unhandled (10-t & Flow-through)	Control temperature (28.5°C / 27.5°C)	Control salinity (36‰ / 35‰)	Artificial	Cont –T28 S36
		Handled (2-t & Static-water)	Control temperature (28.5°C / 27.5°C)	Control salinity (36‰ / 35‰)	Artificial	T28 S36
		Handled (2-t & Static-water)	Control temperature (28.5°C / 27.5°C)	Control salinity (36‰ / 10‰)	Artificial	T28 S10
		Handled (2-t & Static-water)	Control temperature (28.5°C / 27.5°C)	Control salinity (50‰ / 35‰)	Artificial	T28 S50
		Handled (2-t & Static-water)	Control temperature (35.5°C / 27.5°C)	Control salinity (36‰ / 35‰)	Artificial	T35 S36
		Handled (2-t & Static-water)	Control temperature (35.5°C / 27.5°C)	Control salinity (36‰ / 10‰)	Artificial	T35 S10
		Handled (2-t & Static-water)	Control temperature (35.5°C / 27.5°C)	Control salinity (50‰ / 35‰)	Artificial	T35 S50

[1] Handled means prawns have been handled and transferred to the 'stress' tanks; Unhandled means prawns remain in their holding tank throughout the 'stress' period

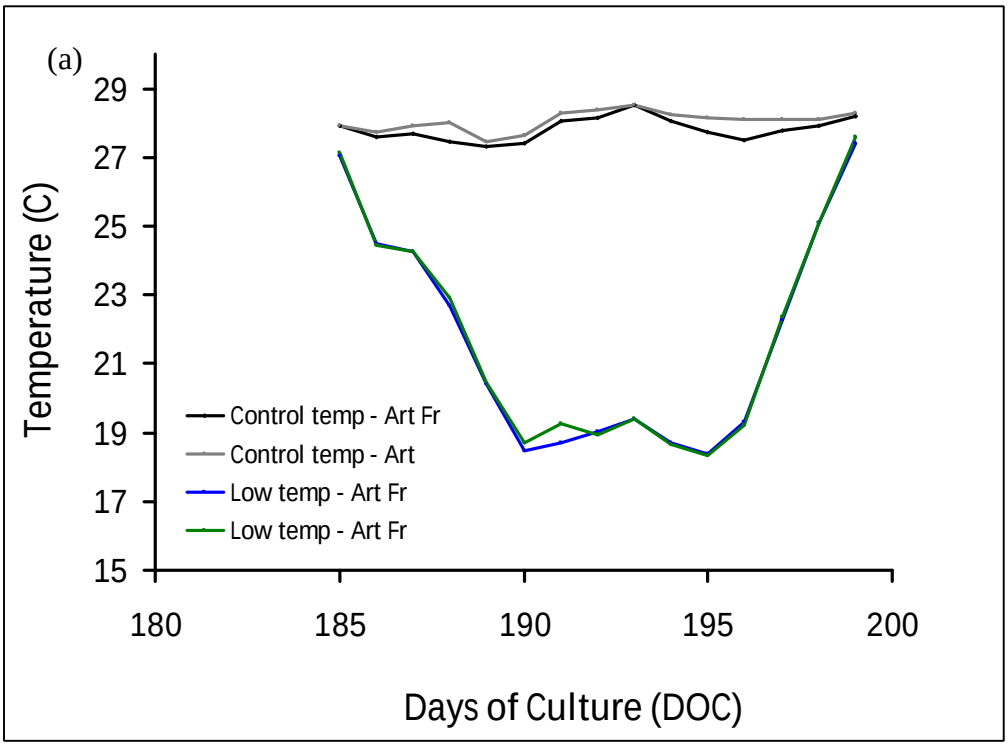
[2] Nominal mean values presented; actual maximum and minimum temperature profiles for the 'stress' period are presented in Appendix 1.5 and 1.6

[3] Nominal mean values presented; actual maximum and minimum salinity profiles for the 'stress' period are presented in Appendix 1.6

[4] Codes used relate to treatment presented in Appendix 1.4

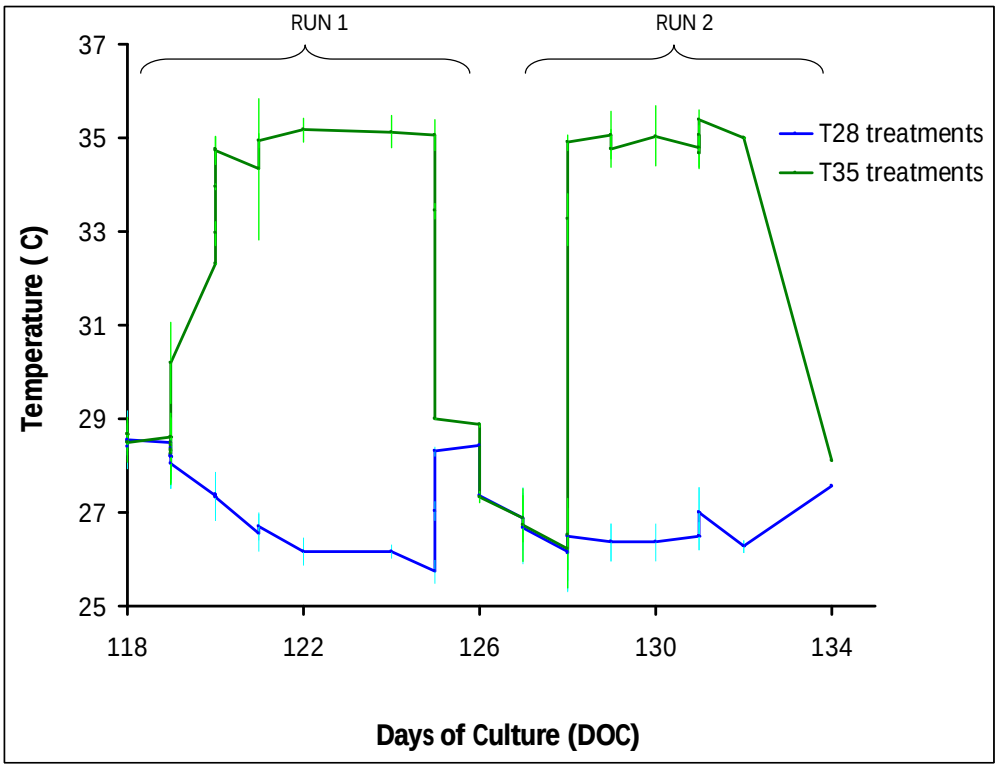
Appendix 1.5

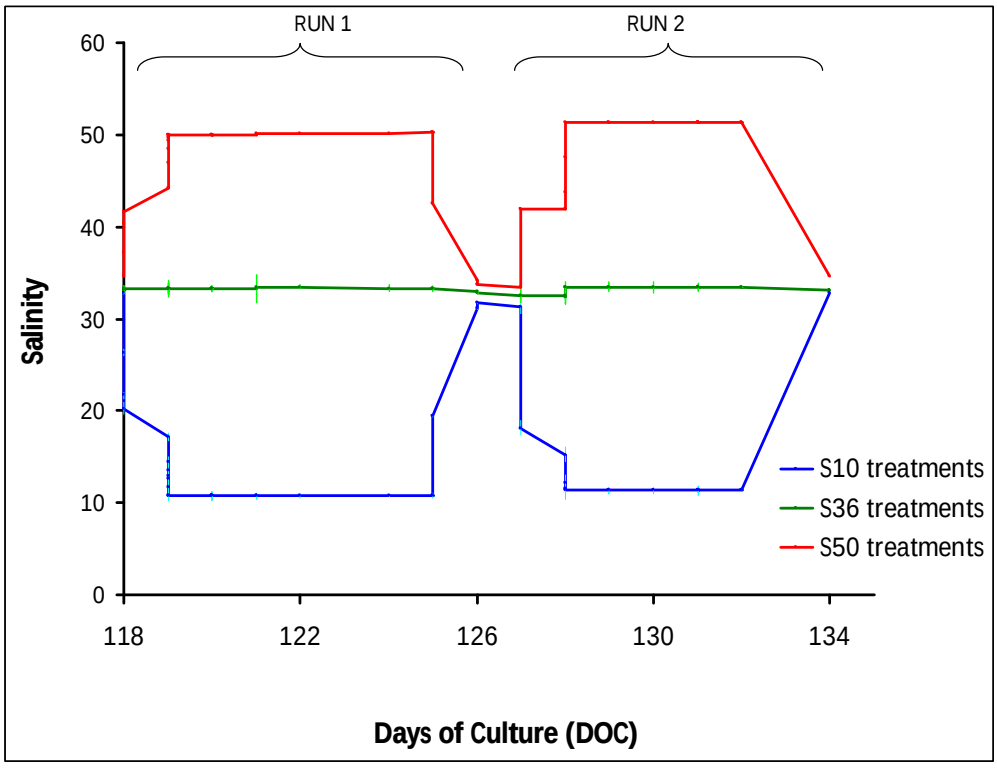
Figures of temperature profiles for each of the 'handled' temperature treatments in the first years' tank trial (i.e. low temperature – diet trial).



Appendix 1.6

Figure of mean (± SD) temperature and salinity profiles for the 'handled treatments', across the two 'runs', in the second years' tank trial (i.e. high temperature – salinity trial); presented as averages for each respective stress treatment.





Appendix 2: Male fertility in Penaeus monodon – A manual for hatchery operators

Increasing seedstock production of domesticated giant tiger prawns (*Penaeus monodon*)

Project No. 2008/756

APPENDIX 2 – Male fertility in Penaeus monodon – A manual for hatchery operators

The Australian Seafood CRC is established and supported under the Australian Government's Cooperative Research Centres Programme. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.

Australian Government
Fisheries Research and Development Corporation

Male fertility in *Penaeus monodon* – A manual for hatchery operators.

The purpose of this manual is to provide commercial famers of *P. monodon* with a reference guide to male fertility. The first component of the manual provides a "step-by-step" list of recommendations to help farmers determine whether fertility issues within the hatchery are related to male broodstock and then identify the likely cause of the fertility problem. The second component then discusses the practicality and reliability of the various measures of male fertility that are available. The manual has been constructed based on information obtained from the CRC Male Fertility project, information from other reproductive experiments conducted at CSIRO, general consensus in the literature and consultation with industry partners. Importantly, this manual can only provide advice from what is currently known of male fertility in *P. monodon*. Therefore, this manual should be considered as a "work in progress" and updated as new knowledge is learned.

1. Identifying male fertility problems in the hatchery

Based on our knowledge of measures of male fertility to date; there are some fertility problems that occur within the hatchery that we can diagnose with a high degree of reliability and others where the cause is still uncertain. The accuracy of the diagnosis when fertility problems occur in the hatchery will be influenced by the nature of the problem, the time required for diagnosis and the resources available. With this in mind we have developed the following flow chart that summarises the links between observed fertility problems in the hatchery and possible causes (Figure 1). Furthermore, it's important to understand the relevant physiological and behavioural processes involved from mating through to nauplii hatching, therefore Table 1 has been created to compliment the flow chart.

The flow chart begins with the two problems that occur within the hatchery that could potentially be related to male fertility; zero hatching eggs from spawnings and low hatch rate of eggs. It is critical to differentiate between the two because the possible causes can be completely different.

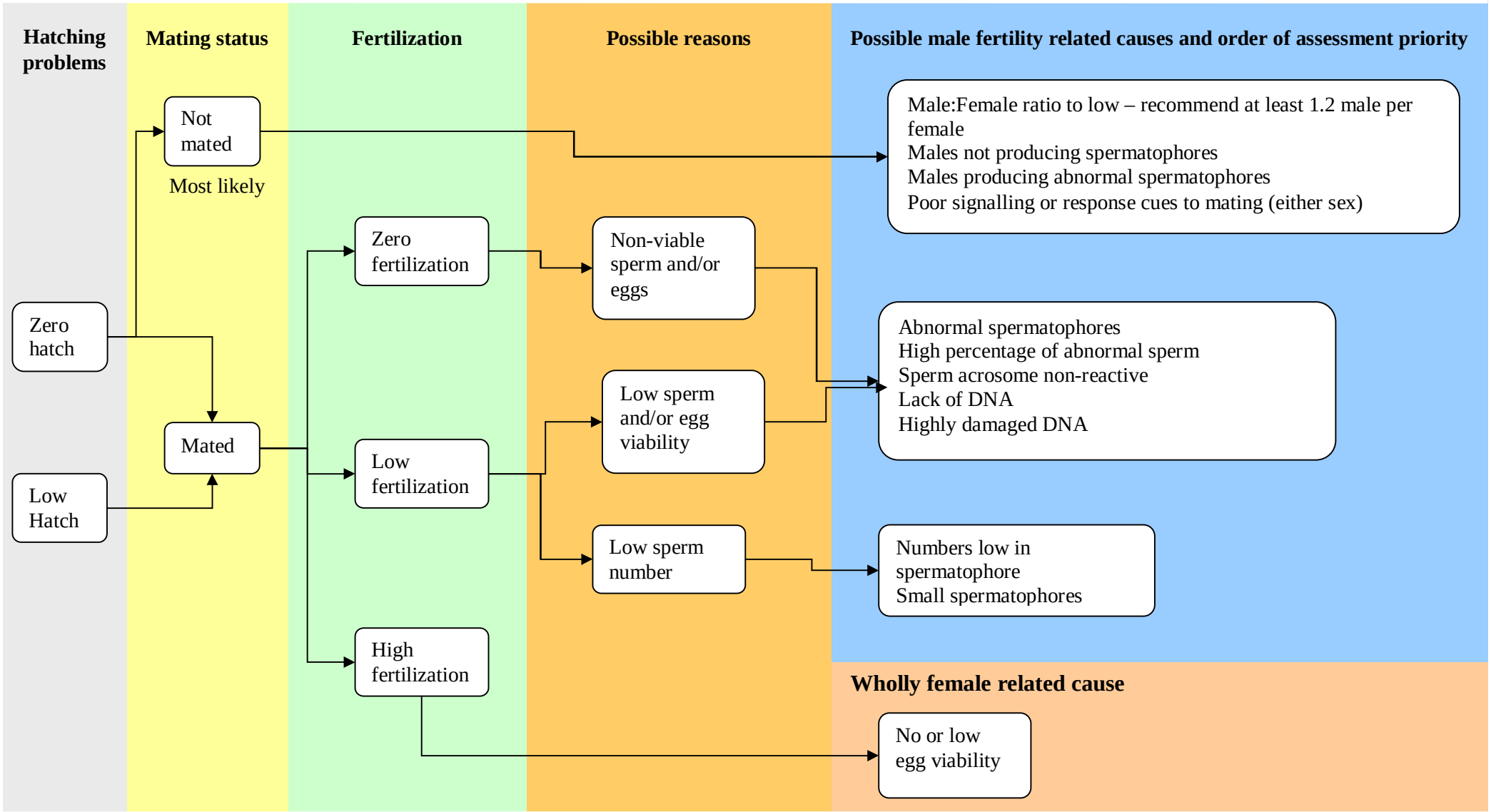


Figure 1. A flow chart reporting two problems observed in the hatchery that are fertility related, the possible reasons for the problem occurring and possible causes related to male and also female fertility.

1.1 Zero hatch of eggs

Determining mating status

When no eggs hatch into nauplii, we believe that the most frequent cause is the female was not mated. Accurately determining the impregnation status of the female is critical because this can indicate completely different causes of the fertility problem. The dilemma is that non-destructive assessment from external observation of the thelycum can be unreliable, especially with untrained technicians. A non-destructive "biopsy" via insertion of a needle into the thelycum can give reliable positive results but also false negatives (i.e. if sperm found, mating is certain but if no sperm found, she was not necessarily unmated). Destructive assessment through dissection of the thelycum is very reliable, but obviously results in loss of stock. If zero hatching is frequent in the hatchery, then sub sampling females for destructive assessment would be recommended to improve the diagnosis. Importantly, before any assessment is undertaken, it is critical to be absolutely certain that the spawn was zero hatch, as even 1 or 2 nauplii within the spawning tank can indicate that the female was actually impregnated.

A non-mated female

An insufficient number of reproductively "capable" males is likely to be one of the primary reasons for non-mated females. A ratio of at least 1 male per female is typical practice, but we recommend a ratio of 1.2:1 or higher to improve chances of mating success. It is therefore important to keep ongoing records of the sex ratios in the tanks. Non-mating could also occur when male numbers are sufficient, but not all are reproductively "capable". This could occur when males aren't producing spermatophores or the spermatophores are so severely abnormal (Fig. 6B) that they are unlikely to be able to mate. All these scenarios can be easily checked, and should be the first assessments conducted when zero hatch rates are occurring. However, if the males are in adequate numbers and producing normal spermatophores, another possibility is that the signalling and response cues to mating are inadequate from either sex (Marsden, 2008). Males should be observed actively pursuing females when they moult and also make frequent attempts to position himself under her (Fig. 3).

A mated female

If the female was mated then it's important to know the fertilization rate (Fig. 4) post spawn as this can help narrow down the possible causes. If fertilization was high, the cause is highly likely to be a lack of embryo development; which is believed an almost entirely egg-related problem (general consensus in the literature and our own results (to be published)). Therefore, it's more relevant to assess the condition of the female broodstock rather than the males. If fertilization was zero, then the problem could be either non-viable sperm or non-viable eggs or a combination. There are a number of possible male related causes of non-viable sperm, but these are discussed below as they also relate to causes of low hatch rates.

1.2 Low hatch rate of eggs

A low hatch is different from a zero hatch because we know the female was impregnated. Therefore the cause of a low hatch can be one or a combination of three fertility problems; low sperm numbers, low/non-viable sperm, or non-viable eggs. We recommend taking the following steps, starting with the most practical, to determine if a problem with the males is a possible cause.

External observation of the male genitalia

An external observation of the male genitalia is very easy to perform and can be used to identify any obvious problems with spermatophores (see section 2.1).

Spermatophore morphology

Spermatophores can be easily removed from the male via artificial ejaculation using an electrostimulant (3.5V(AC) for 1-4sec duration). CSIRO/DEEDI use a 9V(DC) battery with a converter (Fig. 2A), place the electrodes anterior and posterior of the terminal ampoule and apply gentle inward pressure with the thumb and forefinger (Fig. 2B). Spermatophores can easily be assessed for normal morphology/colouration (Fig. 6A). Males with severely abnormal spermatophores (Fig. 6B) may not even be able to mate and can be reliably considered as infertile. However, it's important to look for subtle anomalies in morphology (see section 2.2) as they could also indicate compromised reproduction, such as low sperm numbers or non-viable sperm.

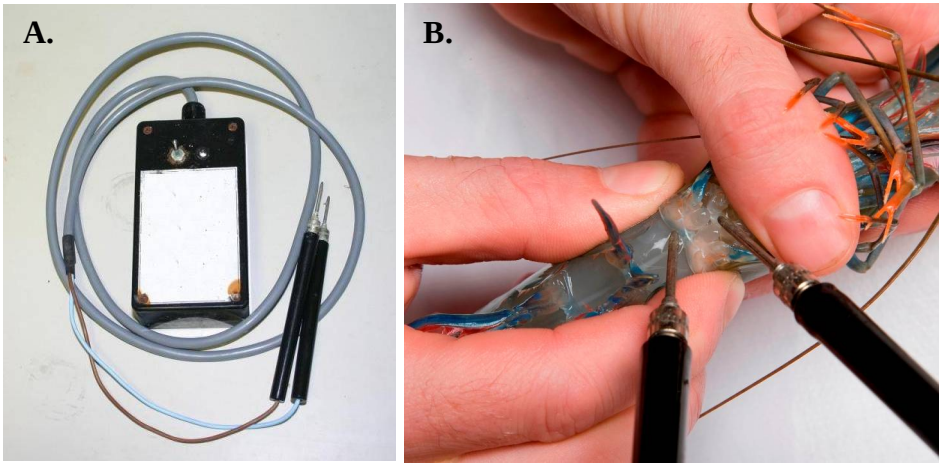


Figure 2. A. Electrostimulant device. B. Technique for artificial extraction of spermatophores using an electrostimulant.

Spermatophore size

Prior to placing spermatophores in seawater for morphological assessment, they should be weighed immediately post removal from the terminal ampoule. Along with obtaining the male body weight, this will allow comparison with the spermatophore weights in Figure 7, section 2.3. As a rough guide, any spermatophores well below the blue line could indicate a male with sub-optimal fertility potential and possibly low sperm numbers. For example, an 80g male should produce spermatophores in the range of 30-90mg.

Sperm number and morphology

If spermatophores are normal size and morphology, then the next step is to evaluate the sperm under light microscopy (400X). Appendix A outlines a protocol for accurately estimating sperm numbers and the percent of abnormal sperm. Sperm numbers for a given size spermatophore can be compared against the data in Figure 8, section 2.4. Anything well below the blue line indicates substantially low sperm numbers and could potentially contribute to low fertilization rates. For example, a 60mg spermatophore typically contains between 40 and 150million sperm cells.

Sperm morphology is reasonably easy to assess using the same sample used to estimate sperm number. Again, the percent of abnormal sperm can be compared against the data in Figure 9, section 2.4. Anything well below the blue line could indicate compromised sperm viability and potentially contribute to low fertilization rates. On average, spermatophores contained 73.4% normal sperm (blue line) and 90% of spermatophores had greater than 50% normal sperm (red line). Another potential sign of low sperm viability are contaminated samples, identified by the presence of ciliates and flagellates swimming amongst the sperm (Fig. 9C).

Sperm viability

In situations where spermatophores are normal and the number of sperm and percentages of sperm with normal morphology are high, then it would be important to consider potential problems with egg viability. However, if there are no reasons to suspect problems with the eggs, then the next recommended male fertility assessments would require vastly more complex and time consuming procedures. Our results have demonstrated that the acrosome reaction assay has the most potential for identifying sperm viability problems. However, the method is complex and requires invasive/destructive sampling of females to obtain matured sperm from the thelycum, equipment to collect and store egg water and also an experienced technician to interpret the results. Therefore, it would be our recommendation to a commercial hatchery wishing to develop this capability, to do so in direct consultation with CSIRO/DEEDI, as the level of detail involved is beyond the scope of this manual (see section 2.5 for further details).

While we reported no obvious association with the comet assay and egg fertilization, it is important to consider that the sperm DNA damage was fairly low in all the males assessed. High levels of DNA damage have been linked to fertility problems in other species, so if high levels of DNA damage were reported from prawn broodstock with fertility problems, it would not be unreasonable to suspect a link. In the immediate future, the comet assay will likely remain as an important research tool for improving our understanding of sperm viability in *P. monodon*.

Table 1. Steps from mating of broodstock to nauplii hatching

Step ***(event)***	**Relevant physiology & behaviours**	**Status of propagules** ***(normal circumstances)***
1. Mating	* Males sense female is about to moult * Female moults and is pursued by males * Male clasps onto the underside of female (Figure 3A and B) and transfers two 'twin' spermatophores *(packets of sperm)* into the females thelycum *(receptacle)*.	* Eggs immature in female * Sperm *(within the spermatophore)* are less able to undergo an acrosome reaction
2. Propagule maturation in the female	* The eggs mature within the female's ovary *(located in the female's abdomen and carapace)* * The sperm matures within the female's thelycum * Progressively, the spermatophores break down within the thelycum until they are no longer discrete structures	* Egg develop to maturity * Sperm capacitation occurs, increasing their ability to undergo and acrosome reaction
3. Spawning and fertilisation	* Mature eggs are released by the female into an "eddy" adjacent to the female's gonopore *(i.e. the eddy is outside her body)* * At the same time, matured sperm are released from the thelycum of the female *(the mechanisms controlling this release are not known)* into the "eddy" * Eggs are 'activated' when they hit the salt water, allowing a small period of time *(approx. 1 minute)* at which the eggs are receptive to fertilisation by the sperm *(i.e. before the egg hatching envelop forms)* * Eggs and sperm are rapidly mixed in the female's "eddy" before flowing into the water column * Sperm attach to the egg and undergo and acrosomal activation to fertilize the egg * Fertilization occurs within a minute post release of the eggs	* Eggs are mature * Sperm is mature
4. Embryo development †	* The fertilised eggs *(now termed embryos)* develop through a series of cell divisions * The first cell division *(i.e. a division from 1 to 2 cells - termed "2 cell")* occurs approx. 40 minutes after spawning (Fig. 4B) * The following cell divisions occur every approx. 20 minutes subsequently * Embryos develop through to nauplii *(i.e. the first larval stage hatching from the within the embryonic cell)* by 12 hours post spawning	* Eggs are fertilised by a single sperm *(i.e. fertilisation by more than one sperm, termed polyspermy, is inhibited)* * Embryos develop through to nauplii

† Egg fertilisation is typically assessed at the "4 cell" stage *(which is approx. 1 hour post spawning)*

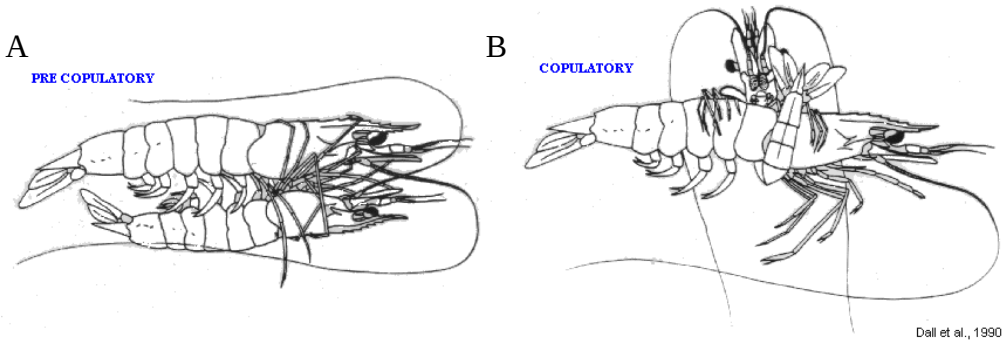


Figure 3. Diagram of pre copulatory (A) and copulatory (B) mating positions.

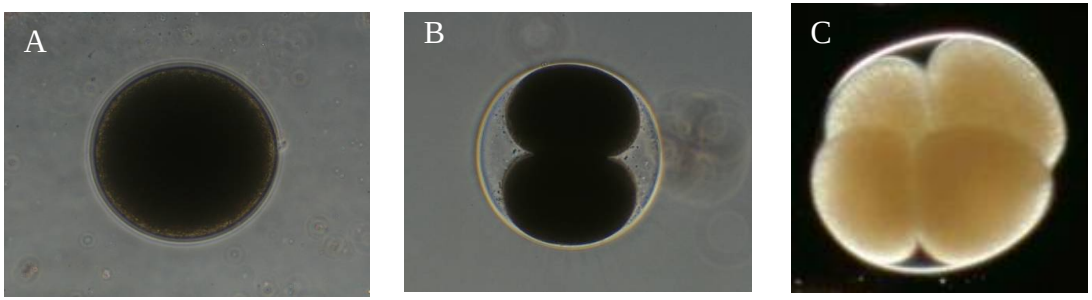


Figure 4. Photo of a 1-cell embryo (A), 2-cell embryo (B) and 4-cell embryo (C) as viewed under light microscopy.

2. Measuring male fertility

The measure of an individual male's "fertility" can be simplified to the ability of his propagules to fertilize an egg. However, this fertilizing ability is determined by the combined condition and function of various anatomical and physiological components. Broadly, the reproductive system (testes, vas deferens and terminal ampoule) needs to be fully developed and anatomically normal. This system then needs to perform all the required physical and biochemical functions to produce normal spermatophores within the terminal ampoule packaged with a sufficient number of viable sperm cells. Once ready for mating, a male then needs to respond to physiological cues from a female and successfully transfer two spermatophores into the female's receptacle (thelycum). Failure at any point along this process can lead to partial or even complete infertility on the male's behalf. In order to develop a practical measure of fertility, it is therefore important to not only consider the condition and function of these various components but also the complexity of the methods and extent to which they can be reliably assessed.

Practicality of measures

Numerous methods have been evaluated for assessing the various components that influence fertility. These methods range in practicality from simple observational and quantitative assessments of spermatophore morphology and weight to the highly detailed measure of DNA fragmentation within individual sperm cells. Table 2 outlines the details of the measures that were evaluated and categorises them into three levels of practicality; high, medium and low. Practicality is based on the required equipment, trained technicians and sample type (e.g. sperm from a spermatophore (S-sperm) sample is far more practical than sperm from a female thelycum (T-sperm)).

Reliability of measures

Evaluations also involved determining the reliability of methods for predicting fertility, generally focussing on the most practical measures first. We found that for any given component of fertility that is assessed, the degree of reliability often depends on the outcome. Most methods can reliably determine infertility but their ability to predict high fertilizing potential are less reliable. For example, a melanised spermatophore with severely abnormal morphology can be reliably considered as infertile, whereas a spermatophore with normal morphology is not necessarily fertile because the sperm within the spermatophore may still be non-viable. Reliably identifying 'males of known infertility' is still very important in contributing to the weeding out of poor males from the hatchery operation. However, in terms of reliably identifying highly fertile males, the situation is far more complex due to the difficulty in determining sperm viability.

The reliability of fertility measures is most ideally evaluated based on its relationship to egg fertilization. However, due to the difficulty of performing this correlation, only some measures have been evaluated with this method. Therefore, we had to evaluate the reliability of other measures on a logical assumption based on the results from an experiment examining the "fertilizing capability" of sperm from different males. That experiment found that spermatophores with sufficient numbers of morphologically normal sperm can still produce low fertilization rates even in females with viable eggs, and also that fertilization rates from these males was highly variable. The reliability of the measure was based on the logic that; if there is no variability between males, then it is unlikely that the measure is discriminating in any way. Table 3 outlines all the measures evaluated and summarises their reliability. The following section provides a brief discussion of the practicality and reliability of each measure.

2.1 External observation of the genitalia

An external observation of the male genitalia is a quick and simple method of identifying obvious problems with spermatophores. Normal spermatophores will appear as white colourations at the based of the fifth pair of walking legs inside the terminal ampoule (Fig. 5). Severely melanised spermatophores are obvious, but even slightly discoloured spermatophores can be identified with experience. Importantly, a visual absence of spermatophores does not always indicate an actual absence. Spermatophores can still be artificially ejaculated even when not visually obvious.

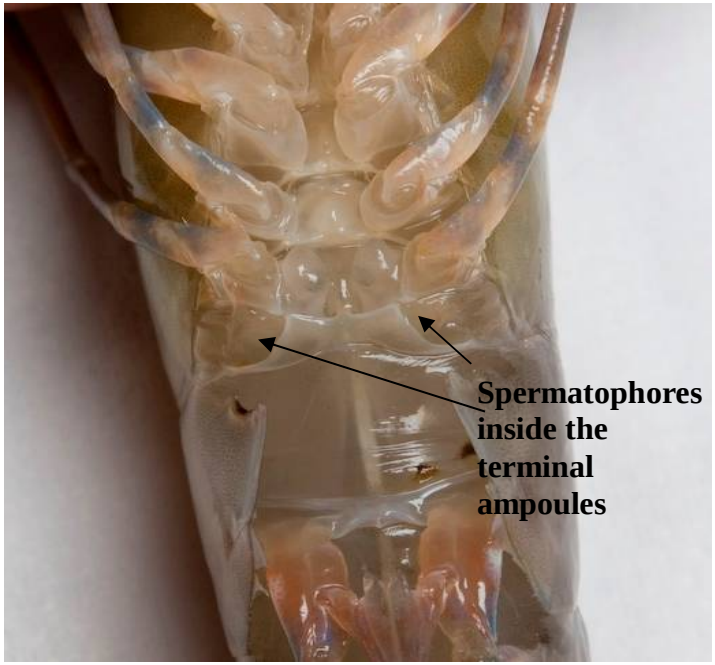


Figure 5. Ventral side view of the male genitalia showing spermatophores visible within the terminal ampoule at the base of the fifth pair of walking legs.

2.2 Gross spermatophore morphology

Assessing spermatophore morphology is a simple diagnostic tool for assessing the reproductive condition of a male. Figure 6A shows the morphology of a normal spermatophore after being submerged in seawater for 5min. Males with severely abnormal spermatophores (Fig. 6B) may not even be able to mate and can be reliably considered as infertile. However, subtle anomalies in morphology could also indicate compromised reproduction, such as low sperm numbers or non-viable sperm. These anomalies include:

- Discoloured (yellow or brown) main body or tail fan (Fig. 6C and E)
- Absence of a tail fan
- Completely opaque main body (the main body should be transparent containing white sperm bundles)
- Completely transparent main body (no sperm bundles)
- Lack of form (Fig. 6D) (the main body should be oval with a defined apex)
- Lack of cohesion (easily breaks apart when gently removed from the terminal ampoule)

It is critical for males to be producing morphologically normal spermatophores to have any chance of being highly fertile. However, the reliability of this measure ends here, as normal spermatophores can still have low sperm numbers or non-viable sperm.

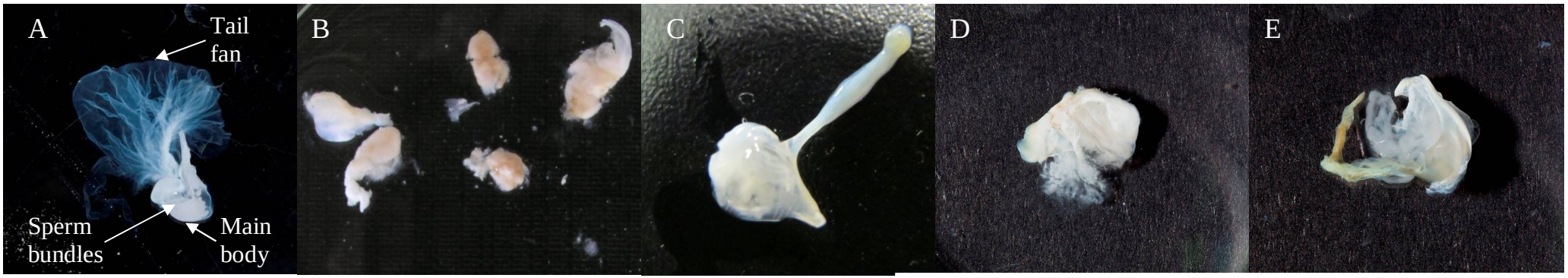


Figure 6. A. Spermatophore unravelled in seawater demonstrating normal morphology and colour. The main body is round at one end and comes to an apex at the other. The main body colour is transparent/white with visible sperm bundles. The tail is white and fans out when immersed in seawater. B. Severely abnormal spermatophores unravelled in seawater. The main body is irregular in shape and brown in colour and the tail is completely absent. C. Slightly discoloured (yellowish) spermatophore immediately after removal from the terminal ampoule. D. Spermatophore with a main body lacking form. E. Spermatophore with a discoloured (yellowish/brown) tail.

2.3 Spermatophore size

Although the “fertilizing capability” experiment demonstrated no significant correlation with spermatophore weight and egg fertilization, it’s important to note that spermatophores used in that experiment were of average size and reasonably uniform across males. Over the past 2 years, CSIRO and DEEDI have collected a large data set of spermatophore weights from a range of stocks of different body weights (Fig. 7). The data demonstrates a significant relationship with body weight and spermatophore weight, but also demonstrates the large variability in spermatophore weight for a given male weight. An arbitrary red line has been placed on the plot to help identify smaller spermatophore weights for a given male weight. Anything well below the red line could indicate a sub-optimal spermatophore and possibly low sperm numbers. However, as for morphology, a normal size spermatophore can still contain low sperm numbers or non-viable sperm.

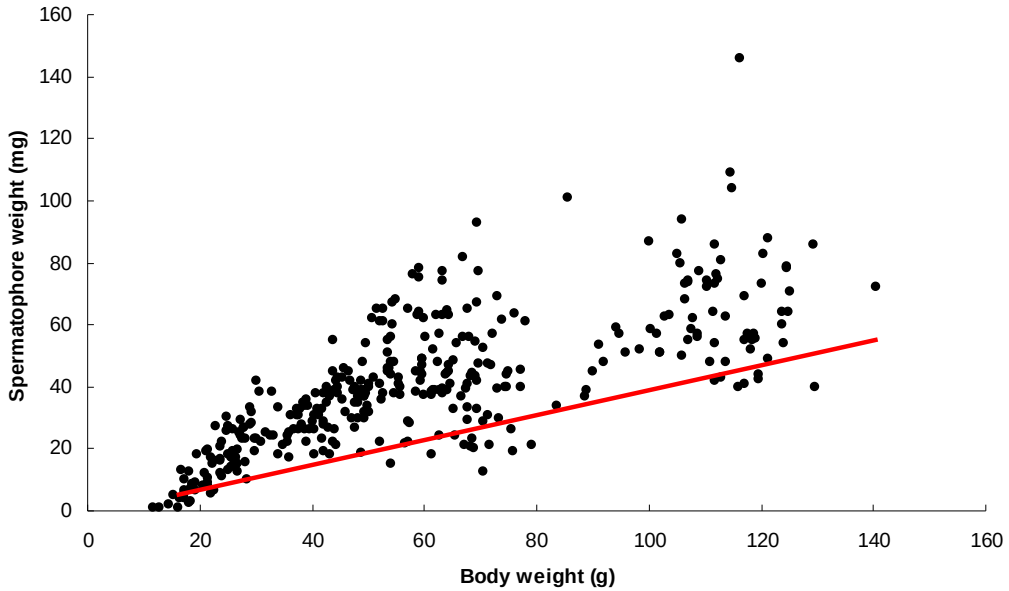


Figure 7. Body weight (g) and spermatophore weight (mg) from *Penaeus monodon* males collected from stocks reared at both CSIRO and DEEDI during the male fertility project and other research projects. This includes data from wild, G1, G3 and G8 broodstock. An arbitrary red line helps to identify smaller spermatophore weights for a given male weight. Spermatophores well below the red line could indicate a sub-optimal spermatophore and possibly low sperm numbers.

2.4 Sperm number and morphology

Again, although the “fertilizing capability” experiment found no significant correlation with sperm number/morphology and egg fertilization, there are two important points to consider from that experiment. Firstly, the four males with the lowest sperm numbers (below 40million) all fertilized a low percentage of eggs (<13%). However, the paired male in three of these matings also fertilized a low percent of eggs so we could not rule out a female problem. Secondly, no males had really low percentages of normal sperm, with the lowest at 47% and average at 76%. Therefore, we cannot rule out that low sperm numbers and low percentages of normal sperm, have no impact on egg fertilization. Figure 8 shows a plot of sperm numbers recorded from *P. monodon* spermatophores at CSIRO and DEEDI over the past 2 years. An arbitrary red line has been placed on the plot to help identify lower sperm numbers for a given spermatophore size. Anything well below the red line indicates substantially low sperm numbers that could potentially contribute to low fertilization rates.

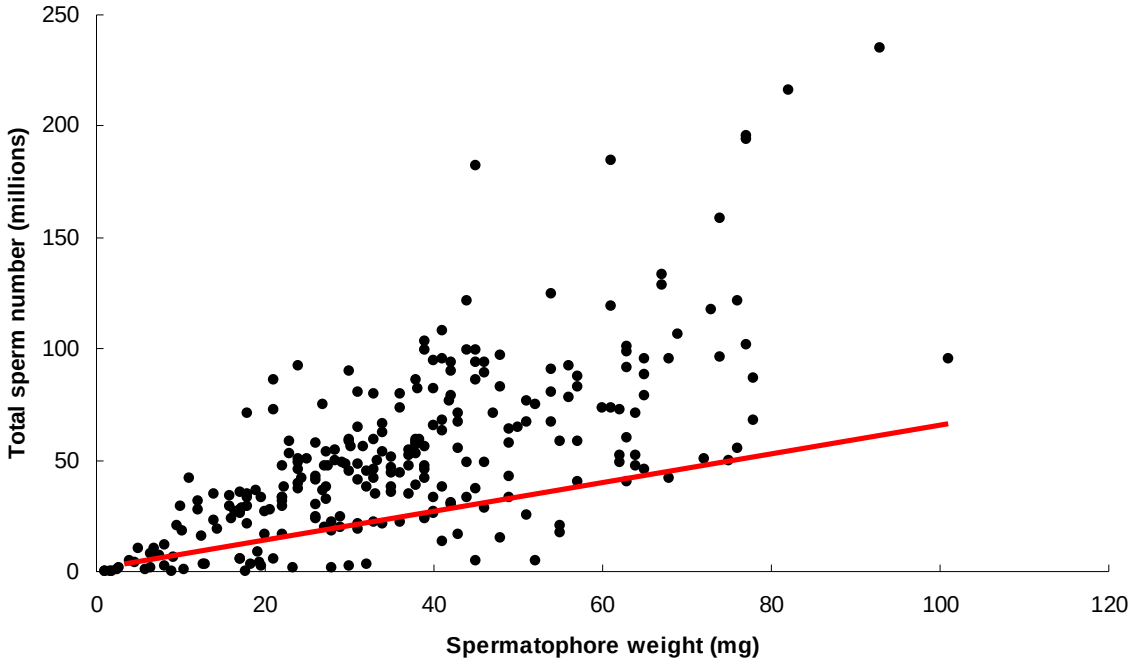


Figure 8. Spermatophore weight (mg) and sperm number (millions) from *Penaeus monodon* males collected from stocks reared at both CSIRO and DEEDI during the male fertility project and other research projects. This includes data from wild, G1, G3 and G8 broodstock. An arbitrary red line helps identify lower sperm numbers for a given spermatophore size. Anything well below the red line indicates substantially low sperm numbers that could potentially contribute to low fertilization rates.

Sperm morphology is reasonably easy to assess. Normal sperm have a round main body with reasonably straight spike (acrosome)(Fig. 9A). Abnormal sperm can show a range of anomalies including; shortened spike, small main body, irregular body shape, bent spike, and missing spike (Fig. 9A and B). Figure 10 shows a plot of the percent of normal sperm recorded from *P. monodon* spermatophores at CSIRO and DEEDI over the past 2 years. On average, spermatophores contained 73.4% normal sperm (blue line) and 90% of spermatophores had greater than 50% normal sperm (red line). Anything well below the red line could indicate compromised sperm viability and potentially contribute to low fertilization rates. Another potential sign of low sperm viability are contaminated samples, identified by the presence of ciliates and flagellates swimming amongst the sperm (Figure 9C).

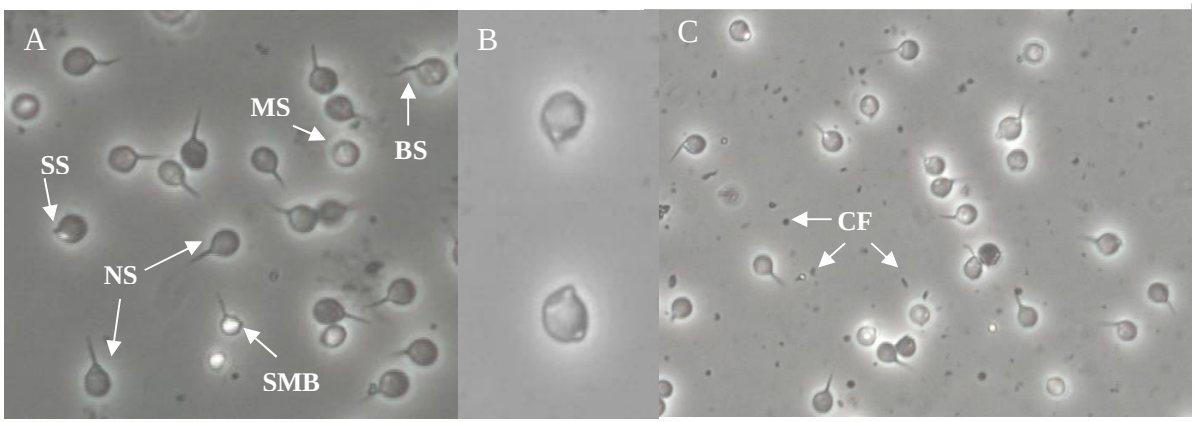


Figure 9. A. Sperm under light microscopy at 400X showing normal sperm (NS), and abnormal sperm; shortened spike (SS), small main body (SMB), bent spike (BS) and missing spike (MS). B. Sperm with missing and shortened spike observed under light microscopy at 1000X. C. A contaminated sperm sample at 400X showing numerous ciliates and flagellates (CF), often observed when spermatophores are abnormal (mostly when discoloured).

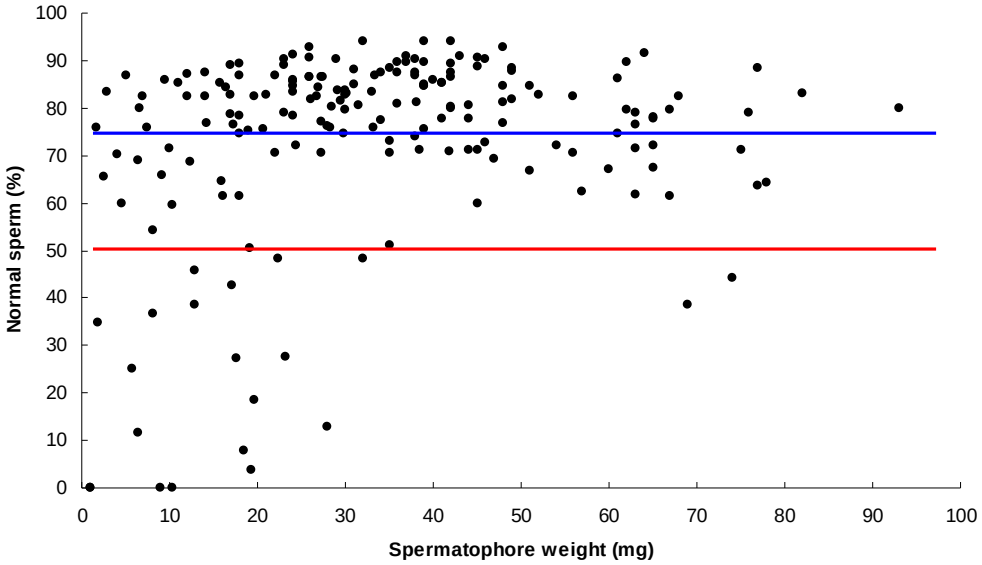


Figure 10. Spermatophore weight (mg) and 'normal' sperm (%) from *Penaeus monodon* males collected from stocks reared at both CSIRO and DEEDI during the male fertility project and other research projects. This includes data from wild, G1, G3 and G8 broodstock. On average, spermatophores contained 73.4% normal sperm (blue line) and 90% of spermatophores had greater than 50% normal sperm (red line).

2.5 Sperm viability

Acrosome reaction

This is generally considered as the most reliable measure of sperm viability (even thought previous research has never established the association with egg fertilization) due to it being an essential requirement for fertilization. However, our initial evaluations didn't focus on this measure due to being considered one of the least amenable in the commercial setting. The measure is only reliable on sperm that has matured within the thelycum (T-sperm), which can only be obtained invasively via a needle inserted into the thelycum or destructively via dissection of the thelycum. Consequently the outcome can only be related back to an individual male if the female was artificially inseminated. Furthermore, the only stimulant known to react the sperm is natural egg water, which requires collecting the proteins released from the eggs immediately post spawning, processing the proteins with a centrifuge and then storage in liquid nitrogen. While the assay is fairly simple to perform, it requires an experienced technician to evaluate the reactive status of cells (Fig. 11).

However, due to the unreliability of other measures evaluated, further research was conducted on the acrosomal reaction. An attempt was made to increase the practicality of the assay by testing an artificial stimulant (Calcium ionophore) known to cause an AR in other crustacean sperm, but unfortunately this ionophore proved incompatible to *P. monodon* sperm. Natural egg water was then used to assess the variability of AR across a range of males from three different origins (wild and 3rd and 8th generation domesticated stocks) and also related to egg fertilization in wild and G8 stocks. The AR response of sperm was considerably variable between males, the first indication of a reliable measure. When related to egg fertilization from spawnings, the association was fairly strong. These results further support the general thinking that AR is the most reliable measure of sperm viability. However, given the conditions of the assay, the use of AR within the hatchery is restricted. When poor egg fertilization is suspected to be male related, the AR assay could be used on a sample of females T-sperm to assess a cohort of males that were mated with these females. Further research would need to link characteristics of spermatophore sperm to AR potential for the measure to be used commercially to select highly fertile males for the breeding programs.

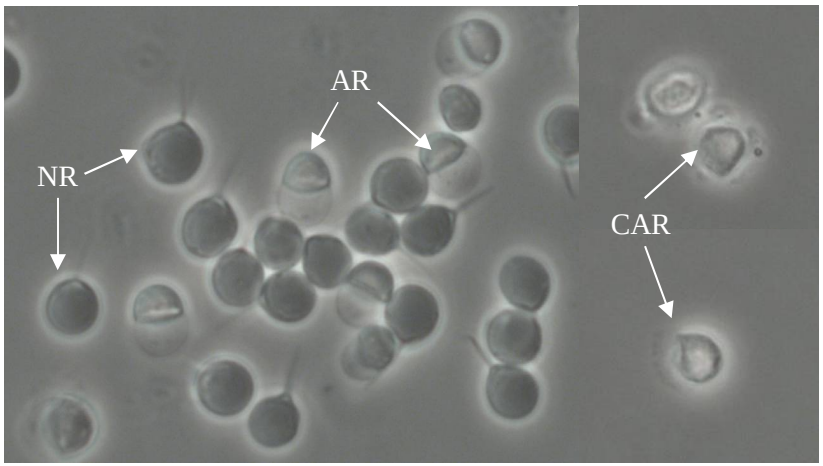


Figure 11. Sperm exposed to egg water *in vitro* showing cells undergoing an Acrosomal Reaction (AR), cells completed an AR (CAR) and non-reactive cells (NR).

DNA fragmentation

Measuring DNA fragmentation using the comet assay requires specialised laboratory equipment and trained technicians to prepare the cells so that the level of DNA fragmentation (Fig. 12) can be evaluated. The comet assay identified some variability between males and also considerable variability between different stocks of males (wild v domesticated). The assay was also sensitive enough to detect DNA damage in sperm that had been exposed to UV for different durations. However, when the comet assay was correlated with egg fertilization, there was no detectable association. It's important to note, that of the 15 spawnings assessed, the DNA damage was relatively low in all males and also the influence of egg quality was not standardised. Therefore, our results can't reliably conclude that DNA damage in sperm does not influence egg fertilization. However, this measure is very suitable for identifying problems with DNA damage in sperm and will be an important tool for future research on *P. monodon* sperm viability.

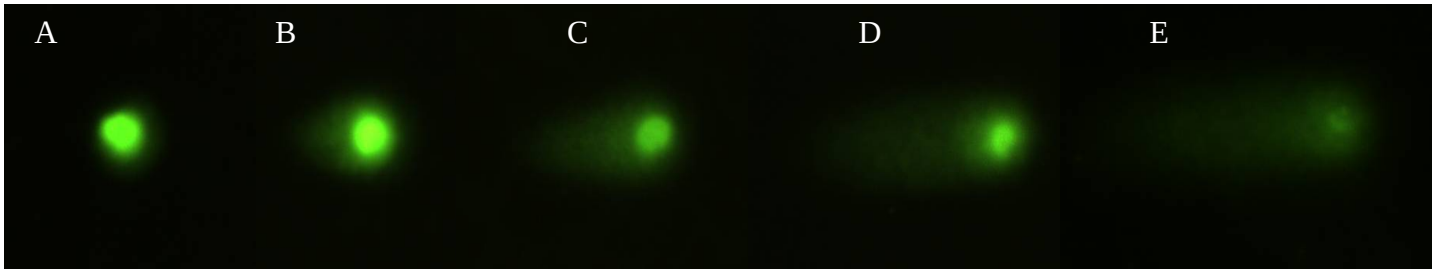


Figure 12. Sperm cells subjected to the comet assay and viewed under a fluorescence microscope (400X) expressing the range of DNA fragmentation scores. A. Grade 0, no DNA damage. B. Grade 1, <20% DNA in the tail. C. Grade 2, 20-50%. D. Grade 3, 50-70%. E. Grade 4, >70%.

Histology of the spermatophore

Five males were assessed from four different stocks (wild and domesticated (G1, G3 and G8)). There was little variation in sperm assessments between males from all stocks except G3. However, G3 stocks had poor quality spermatophores that were easily assessed with the naked eye. There was also no obvious association with histological evaluations of sperm from spermatophores removed from the female thelycum post spawning and egg fertilization. But, this data requires cautious interpretation as there were only 7 spawnings measured. Therefore, it is still unknown whether histology of spermatophores can be used as a tool for evaluating the fertilization capability of a male. However, histology continues to be a useful diagnostic tool when severe incidences of male fertility are observed within the hatchery, such as abnormal spermatophores.

Membrane integrity and mitochondrial function

Cellular stains that evaluate membrane integrity were considered unreliable because there was no variability; all the males assessed had an extremely high percentage (>95%) of cells with intact membranes. In addition, they only identified cells with obvious membrane degradation, which could be more simply identified under light microscopy. Assessing mitochondrial function was considered unreliable because all males assessed had a high percentage of sperm (>98%) that were apparently "functional".

Table 2. Potential measures which could be used to evaluate 'fertility'

Measure	Details of assays used
High practicality	
1. Egg fertilisation	Quantification of the percentage of spawned eggs/embryos undergoing mitotic divisions approx. 1 hour post- spawning *(i.e. at "4 cell")* observed under a light microscope (Fig. 4C).
2. Gross spermatophore morphology	Evaluation of the normality in morphology of the spermatophore structure when ejaculated from a male and allowed to unravel when placed in seawater (Fig. 6A).
3. Spermatophore weight and spermatophore somatic index (SSI)	Evaluation of spermatophore weight on its own and in relation to the size of the male (spermatophore somatic index).
4. Sperm number	Quantification of the numbers of sperm present through a process of cutting and grinding of spermatophores to release the sperm; sub sampling of the resulting sperm solution; and counting of sperm cells within a haemocytometer under the light microscope
5. Gross sperm cell morphology	General observation of S-sperm morphology and quantification of normal versus abnormal cells under the light microscope (Fig. 9)
Medium practicality	
6. Sperm membrane integrity +	Observation of S-sperm cell colour (red or green) after specific cellular staining with SYBR-14 and Propidium iodide (PI), as observed manually under a fluorescence microscope or using an automated reader of fluorescence intensity - FACS (Fluorescence activated cell sorting). Cells are classified as live (intact membrane) or dead (damaged membranes).
7. Presumed mitochondrial function of sperm and membrane integrity	Observation of S-sperm cell colour (red or green) after specific cellular staining with Rhodamine 123 and Propidium iodide (PI) as observed manually under a fluorescence microscope or using an automated reader of fluorescence intensity - FACS (Fluorescence activated cell sorting). Cells are classified as live (functioning mitochondria and intact membrane), dying (non-functioning mitochondria and intact membrane) or dead (non-functioning and damaged membranes).
8. Acrosomal reactivity of sperm cells	Quantification of the percentage of the T-sperm that have 'reacted' after exposure to a 'stimulatory compound' *in vitro* then observed under a light microscope (Fig. 11). The most typical stimulatory compound is egg water, which is the collection of proteinaceous compounds released by the eggs during activation. Artificial stimulatory compounds are yet to exist for prawn sperm.
Low practicality	
9. Histological sperm cell morphology	Evaluation of sperm cell morphology (S & T sperm) in histological sections observed under the light microscope.
10. DNA quantity and quality	Evaluation of the quantity of DNA within a population of S-sperm cells as determined using a spectrophotometer (Nanodrop®)

within sperm cell populations	and quality of DNA within a population of sperm using gel electrophoresis.
11. DNA damage within the sperm cells	Evaluation of the degree of DNA fragmentation within individual sperm cells (S & T sperm) as determined by the Single Cell Gel Electrophoresis assay (comet assay). It involves cell encapsulation in a gel, cell lysis, eletrophoresis, staining with a DNA binding stain (SYBR green I) and measuring of DNA migration (measure of fragmentation) in single cells under a fluorescence microscope (Fig. 12).

High practicality - could be easily conducted on farm as requires minimal equipment and training.
Medium practicality – potentially used on farm with some specialised equipment and/or training.
Low practicality – unlikely to be used on farm as requires highly specialised equipment and trained technicians.
+ Other vital stains such as acridine orange and trypan blue have been used to determine “live” versus “dead” prawn sperm cells. However, there is a tendency for these stains to overestimate the proportion of viable sperm; therefore we did not test these stains within this project

Table 3. **Measures trialled and their reliability to assess male fertility**

Measure	Reliability of the measure for evaluating fertility based on the outcome.
Gross spermatophore morphology	1. Severely abnormal spermatophores are reliable indicators of infertility. 2. Subtle anomalies may indicate compromised fertility. 3. A completely normal spermatophore is very important but does not necessarily indicate high fertility.
Spermatophore weight and spermatophore somatic index (SSI)	1. Not related to egg fertilization when assessed using spermatophores within a typical weight range, and therefore not a completely reliable indicator of fertility. 2. Spermatophores well below the typical range for a given male weight may indicate low sperm numbers
Sperm numbers within the spermatophore	1. Not related to egg fertilization when assessed using sperm numbers within a typical range, and therefore not a completely reliable indicator of fertility. 2. Sperm numbers well below the typical range for a given spermatophore weight, could potentially contribute to low fertilization.
Sperm morphology	1. Not related to egg fertilization when assessed using normal sperm percents within a typical range, and therefore not a completely reliable indicator of fertility. 2. Low percentages of normal sperm well below the typical range could indicate low sperm viability.
Sperm membrane integrity	1. No variability between males and all the males assessed had an extremely high percentage (>95%) of cells with intact membranes. 2. Likely to over estimate the percent of viable sperm.
Sperm mitochondrial function and membrane integrity	1. No variability between males and all the males assessed had an extremely high percentage (>98%) of cells that were classified as “functional”. 2. Likely to over estimate the percent of viable sperm.
Sperm acrosomal activation (using natural egg water)	1. There is large variation between males and also a moderate association with egg fertilization. 2. Likely to be a reasonably good indicator of sperm viability.
Sperm acrosomal activation (using an artificial ionophore)	1. Unable to induce and acrosomal reaction, therefore not a useful measure
Histological sperm morphology	1. Requires further investigation to link histological sperm anomalies with egg fertilization.
DNA quantity and quality within sperm cell populations	1. Current technique not sensitive enough to detect differences between sperm suspensions with known differences in DNA quantity and quality
DNA damage within individual sperm cells	1. Only moderate variation between males for both T-sperm and S-sperm. 2. Damage levels in T-sperm not related to egg fertilization, but DNA damage was minimal in all batches of T-sperm assessed. 3. Detected higher DNA damage in spermatophores with subtle anomalies. 4. Requires further investigation to assess egg fertilization from sperm with higher levels of DNA damage.

* LM = light microscope; EM = electron microscope

Appendix A.

Protocol for estimating sperm number.

1. Add 1.0mL seawater to a ~10mL mixing tube (Fig. 13A).
2. Add whole spermatophore.
3. Thoroughly chop spermatophore into tiny pieces with sharp fine point scissors (Fig. 13B) for ~1min.
4. Use a small cutting tool attached to a low speed power drill to further chop the spermatophore into even finer pieces (~1min). CSIRO typically uses a Dremel drill (Fig. 13C) and small paddle shaped tool (Fig. 13C inset). A standard drill and bit will probably work as well.
5. Add further 2mL of seawater.
6. Seal top with wax paper and vortex (Fig. 13D) for at least 30 sec on high setting. A vortex is preferable, but if unavailable, conduct step 4 for a further minute, seal the tube then vigorously shake. Check replicated counts are consistent to ensure accuracy of the alternative method.
7. Cut the end off 1mL pipette tip (Fig. 13E) and thoroughly homogenise the sample by pumping the pipette.
8. **THE ABOVE STEPS ARE CRITICAL TO ENSURE THAT ALL THE SPERM IS RELEASED FROM THE TISSUE AND THOROUGHLY HOMOGENISED BEFORE LOADING THE HAEMOCYTOMETER. FAILURE TO ACHIEVE THIS WILL LEAD TO VERY INACCURATE SPERM NUMBER ESTIMATES.**
9. Add 10µL to one side of the haemocytometer and do a quick count (see "using a haemocytometer" below).
10. Dilute sample so that there is approximately 40 cells per counting grid. If you have to add more than 7mL, transfer solution to a 20mL mixing tube ensuring that you use the dilution water to rinse out the original 10mL tube.
11. **KEEP ACCURATE RECORDS OF DILUTIONS.**
12. Cover and vortex again for 30sec.
13. Homogenise with pipette.

Using a haemocytometer.

14. Add 10µL to each side of the haemocytometer (Fig. 14A) and leave for 1 min to allow sperm to settle on the bottom (enables easier determination of cell morphology).
15. Prior to viewing, always decide on a specific counting pattern to avoid bias.
16. Count the number of cells in 5 of the 1/25sq.mm grids (Fig. 14B and C) on one side of the haemocytometer and repeat on the other side. Cell morphology (e.g. normal, abnormal, bent spike) can also be recorded for each cell.
17. For each 1/25sq.mm grid pick two walls (e.g. top and left) whereby cells touching these walls are excluded and cells touching the opposing walls are included. Using figure 14C as an example, cells touch the middle line on the top and left are excluded and cells touching the middle line on the bottom and right are included. This would give a sperm count of 51 inside the 1/25sq.mm grid.
18. Each 1/25sq. mm grid has an area of 0.04 mm-squared. The depth of the chamber is 0.1 mm, so the volume of each 1/25sq.mm grid is 0.04 x 0.1 = 0.004 mm-cubed.
19. Therefore, the sperm number per spermatophore equals the mean of counts from the 5 grids/0.004 x dilution amount (in µL). E.g. if the mean of 5 counts is 45 and the spermatophore was diluted in 6.0mL, then sperm count is 45/0.004 x 6000 = 67.5million.

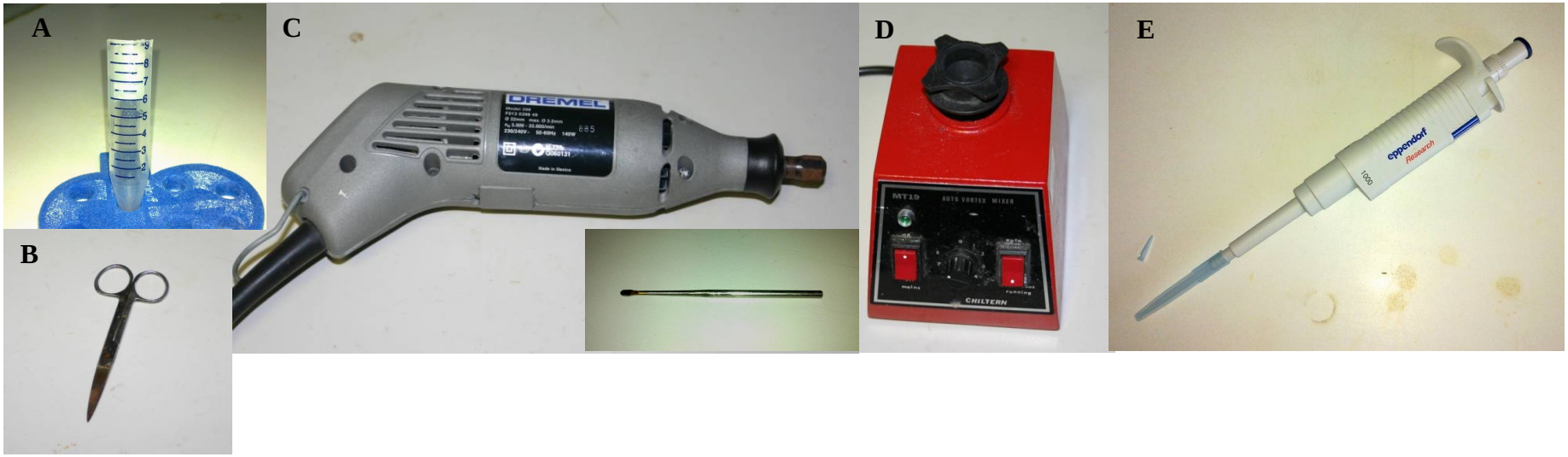


Figure 13. Equipment used in the preparation of sperm suspensions for estimating sperm number and morphology. A. Tube cut to a depth to allow fine point scissors to reach the bottom. B. Fine point scissors. C. Dremel drill and custom made cutting tool (inset). D. Vortex. E. 1mL pipette and tip with end removed.

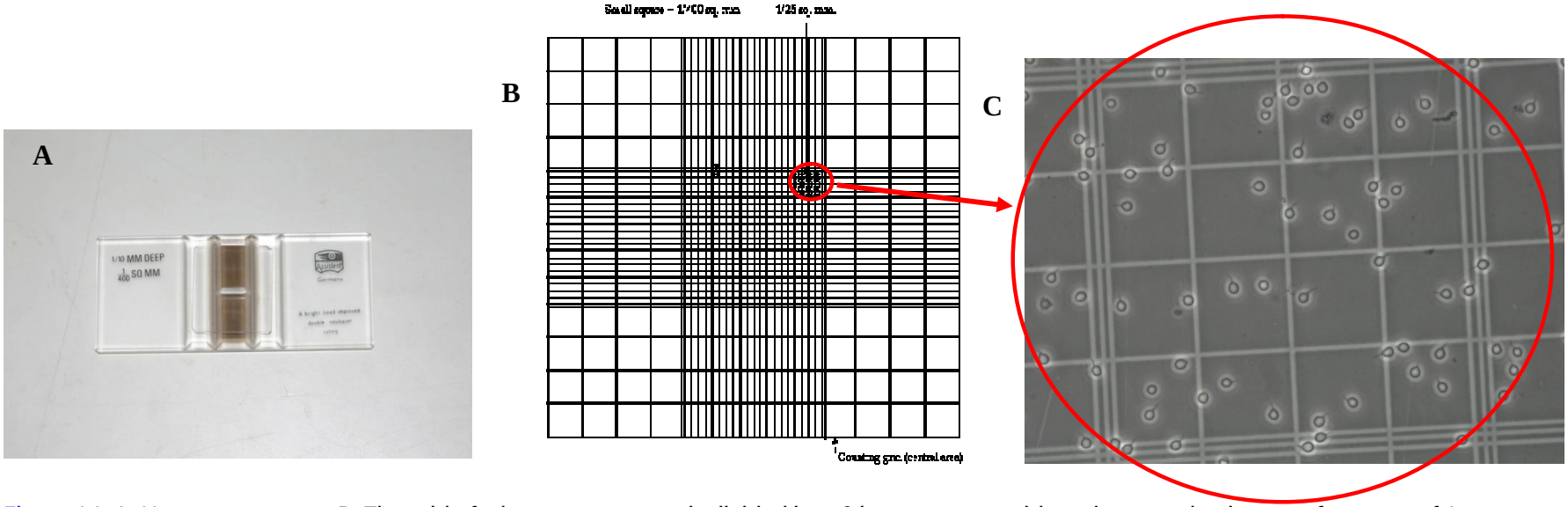


Figure 14. A. Haemocytometer. B. The grid of a haemocytometer is divided into 9 large squares with each square having a surface area of 1 square mm and the depth of the chamber is 0.1 mm. Prawn sperm cells are counted using the 1/25sq.mm grid squares. C. Sperm cells within a 1/25sq.mm grid, which is made up of 16 smaller 1/400sq.mm squares.