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A novel approach to evaluate the relationship between measures of male fertility and egg fertilization in *Penaeus monodon*

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ABSTRACT

Discriminating the male influence on egg fertilization in *Penaeus monodon* is needed so that accurate measures of male fertility can be developed and subsequently used to evaluate methods aimed at improving fertility. In this study, we employed a novel, pairwise comparison approach to discriminating the fertilization influence of males: female broodstock were artificially inseminated with one spermatophore each from a pair of males. We achieved 22 successful 'spawnings' involving selected pairs from 33 males, and estimated the proportion of embryos fertilized by each paired male by individual genotyping of embryos. The non-inseminated, 'twin' spermatophore was used to estimate measures of male fertility; sperm number and the number of normal sperm of each inseminated spermatophore. Relationships between measures of male fertility and egg fertilization were then evaluated. Using this approach we were able to show that the egg fertilization potential of males could not be determined simply by total sperm number or the number of normal sperm (as determined by gross morphology under light microscopy). We also found extremely high variability in fertilization between paired males. The results highlighted male infertility issues (e.g. sperm viability) that are not simply overcome by greater sperm numbers. Our novel approach provides a method that can be used to determine whether more complex measures of male fertility can reliably predict egg fertilization potential.

1. Introduction

Improving the reproductive performance of domesticated broodstock remains a high priority for the black tiger shrimp, *Penaeus monodon*, farming industry in Australia. One of the main issues with many domesticated *P. monodon* stocks is low rates of egg fertilisation, believed attributable in part to compromised male fertility (Pongtippatee et al., 2007). A critical requirement for improving reproductive performance is the development of practical and reliable measures of male fertility, which can be used to determine culturing technologies that optimize fertility. In addition, these measures can be used to remove potential poor performers from commercial breeding programs and monitor the development of stocks over time.

Different methods have been developed to measure various characteristics of male fertility for a number of penaeid species. Some measures, such as spermatophore weight and morphology and sperm number and sperm morphology (Gomes and Honculada-Primavera, 1993; Gupta and Rao, 2000; Jiang et al., 2009; Pratoomchat et al., 1993), are fairly coarse but are simple to perform and therefore practical for the commercial farmer. More complex measures such as vital stains and the acrosome reaction (AR) assay are sometimes considered more reliable because they assess specific characteristics of sperm quality such as membrane integrity (Meunpol et al., 2005; Wang et al., 1995) and ability to activate in contact with egg water (Pongtippatee et al., 2007). However, these measures are less practical for a commercial farmer because they require more specialised equipment and trained laboratory technicians. Regardless of the measure used, relatively little (or nothing at all) is known about the relationship between each different measure and fertilizing capability, which is the ultimate test of fertility. The main constraint to understanding this relationship is the difficulty in discriminating between the male and female influence on egg fertilization.

Understanding the relationship between measures of 'fertility' and fertilization in other aquaculture species is typically approached via *in vitro* fertilization (Au et al., 2002; Casselman et al., 2006; Liley et al., 2002). This method allows gametes from different parents to be mixed and fertilization to be directly compared, essentially discriminating the gender influence. This method has only been achieved for a few spawnings in *P. monodon* (Lin and Ting, 1986) because it is very difficult and constrained by specific reproductive characteristics. In *P. monodon*, sperm undergo a final maturation process within the female receptacle prior to spawning (Vanichviriyakit et al., 2004) and therefore it is difficult to assess 'mature' sperm from the female

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without effecting spawning performance. Also, eggs need to be fertilized immediately post spawning and there is currently no method to induce immediate spawning so that unfertilized eggs can be collected within the required time frame.

The objective of this study is to develop a method that can discriminate the male influence on fertilization so that measures of male fertility can be related to fertilization. Our approach is to artificially inseminate a female with two different males, then compare the proportion of eggs fertilized by each male through individual genotyping. Practical measures of fertility such as total sperm number and sperm morphology will be estimated from the non-inseminated 'twin' spermatophore of each male. Therefore, a prerequisite objective is to determine the accuracy of estimating sperm number and sperm morphology of an inseminated spermatophore, based on measures of its 'twin' spermatophore.

2. Methods

2.1. Broodstock management

Wild broodstock (55 of each sex) caught off the coast of Innisfail (17°53′S, 146°01′E), Queensland, Australia, were obtained through a commercial supplier. The broodstock were stocked into 6×10,000 L tanks with a sand substrate (Crocos and Coman, 1997) at the Cleveland Marine Laboratories (CSIRO Marine and Atmospheric Research, Cleveland, QLD, Australia). Stocking densities were 1.8 m^{-2} and females and males were separated into 3 tanks per sex. Seawater flowed through the tanks at 3.5 L min^{-1} (approximately 50% water exchange day^{-1}). Water temperature was maintained at 29 ± 0.5 °C and salinity at 35 ± 1.0 ppt. Photoperiod was maintained on a 14 h light: 10 h dark cycle with artificial lighting. Broodstock were fed twice daily (0900 and 1600 h) on a diet consisting of approximately (% dry weight) 20% commercial pellets (Lucky Star, Taiwan Hung Kuo Industrial Pty Ltd), 50% squid (*Photololigo* sp.), 20% bivalves (*Plebidonax* sp.) and 10% polychaetes (*Marphysa* sp.). Broodstock were maintained in this system for 3 months during the experiment.

2.2. Estimating total sperm number, and the number of sperm with normal morphology of inseminated spermatophores

Preliminary investigations were performed to evaluate the potential for using the estimate of total sperm number and number of sperm with normal morphology of one spermatophore from a given male as a predictor of the same attributes in the other "twin" spermatophore. The aim was also to investigate the potential of sperm density as a predictor of sperm number in inseminated spermatophores.

Individual males (mean weight of 75.2 ± 13.2 g) were tagged with a coloured and numbered budgerigar tag around one eye stalk and a numbered waterproof paper tag (5 mm × 5 mm) glued (loctite 401) to their carapace in order to track the day of moult. Spermatophores were artificially electroejaculated from males between 10 and 15 d post moult by application of an electrostimulant (3.5 V(AC). We use a 9 V(DC) battery with a converter. All spermatophores were ejaculated within 1–4 secs of applying the stimulant and all males survived the procedure. For 9 individual males, both spermatophores were subjected to a sub sampling and measuring process so that both total number and the number of sperm with normal morphology could be estimated. We used only males with spermatophores of similar morphology and weight (less than 7% difference for all but the smallest pair, which had a 19% weight difference).

To obtain sperm number/morphology data from a given single spermatophore, the spermatophore was first weighed, then placed into a 10 mL plastic mixing tube (Falcon) and 500 μL of filtered (20 μm, UV) natural seawater added. The spermatophore was then chopped into tiny pieces using fine point dissecting scissors. A high speed rotary tool (Model 398-49, carving tool 191, Dremel®, Racine, Wisconsin) was then applied to the solution for 1 min to shred the spermatophore into even smaller pieces. A further 2 mL of filtered seawater was then added to the solution and vortexed (model MT19, Chiltern Scientific) on speed 8 (highest) for 1 min. These steps were critical to release all the sperm cells from the spermatophore tissue and hence produce a solution with homogeneous sperm density. The solution was then diluted with a recorded volume of artificial seawater to produce a sperm cell density that could be easily counted in a haemocytometer. Before being loaded into a haemocytometer, the sperm suspension was further homogenised with a 1 mL pipette (tip cut off). Sperm cell counts per 0.2 × 0.2 mm grid square (0.1 mm depth, volume 0.004 mm^3 or 0.004 μL) ranged from 18 to 136 with a median of 56. Two to three haemocytometers were loaded with sperm suspension. On one side of each haemocytometer, the total count of cells in each of 5 grid squares was recorded. On the other side, two counts were recorded in each of 5 grid squares: the number of normal sperm (spherical main body with spike intact and reasonably straight) and the number of abnormal sperm (malformed bodies or bent, short or missing spikes (Pratoomchat et al., 1993)). For analysis, we summed the 5 grid counts per haemocytometer side, resulting in 4–6 counted subsamples per spermatophore. The proportion of the spermatophore represented by each subsample was thus 0.02 μL divided by the total volume of the spermatophore solution, and this proportion varied from 2.5×10^{-7} to 2.0×10^{-6}, with a median value of 6.67×10^{-7}.

Initial analysis revealed strong correlations in both the total number of sperm and the number of sperm with normal morphology between the "twin" spermatophores (see results Section 3.1), indicating the potential to use this method to estimate attributes of inseminated spermatophores. However, by augmenting this relatively small dataset with all spermatophores from the experiment (including those inseminated, not subsampled), we were able to better define the accuracy of different methods of prediction. This is covered in detail later.

2.3. Artificial insemination

Female broodstock were maintained in the tanks for approximately 2 weeks and were then tagged as per methods for the males and had one eye-stalk ablated. Tanks were monitored daily for moults and females (117.8 ± 15.2 g) were artificially inseminated (AI) 2 d post moult. This time frame was a compromise between the thelycum still being soft enough for insemination but the exoskeleton hard enough to reduce stress during AI. All AI procedures occurred within 7 weeks post ablation.

Two males, 10–20 days post moult, were selected to inseminate a female. Both spermatophores were electroejaculated from each male, then weighed and assessed for morphology. Only males with twin spermatophores of similar weight and morphology were selected for AI (24 of the males used had differences <5%, 15 males <10%, 3 males <15% and 2<19%). One spermatophore from each male was then randomly selected for insemination while the other used to estimate the total number of sperm and the number of sperm with normal morphology. The mean weight of males used for AI was 58.4 ± 6.4 g.

To perform artificial insemination, a female was restrained ventral side up then covered with a wet cloth and her gills kept continually wet. Cotton buds (10 mm × 4 mm ∅) were gently inserted into each side of the thelycum to soak up any fluid. A single spermatophore (tail removed) was inserted into one side of the thelycum with forceps immediately post removal of the cotton buds. The process was repeated for the other spermatophore in the other side of the thelycum. The thelycum was checked to ensure no spermatophores were protruding and the opening had completely closed. The inseminated female was placed in a 100 L holding tank for 3–4 h and observed for loss of spermatophores. The holding tank was black in colour so that lost spermatophores were clearly visible. Only females with no signs of spermatophore loss were included in the experiment.