

An extender solution for the short-term storage of *Penaeus (Litopenaeus) vannamei* sperm and its multiple doses use in artificial insemination

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Introduction

Studies on sperm storage and sperm extenders for penaeid decapods have been mainly addressed to improve cryopreservation. However, short term storage extenders have been used for sperm counting proposes and shrimp hatching laboratories use whole fresh semen for artificial insemination (AI), while nauplii production using AI in *Penaeus (Litopenaeus) vannamei* has been reported but with unknown rate success, although AI technique has been documented.

This study was aimed to develop an extender solution to increase the storage time of fresh sperm, maintaining its function when diluted at several doses for short term storage and to evaluate its AI fertilization capacity in *P. vannamei*, and to test the effectiveness of the AI procedure.



P. vannamei female

Materials and methods

The extender used was Calcium-Free Artificial Sea Water described by Leung and Trujillo (1987) with Antibiotic/Antimycotic (ASW, 822 mOsmol/kg). The percentage of sperm survival was evaluated assessing sperm membrane integrity using LIVE/DEAD® sperm viability kit (Molecular Probes).

Spermatophores were obtained manually (Fig. 1) sperm mass was removed (Fig. 2) and the droplet sits on top of the closed tip of the forceps and rather transferred immediately on the thelycum (Fig. 3) or placed into a 3 mL tube, mixed with ASW and storage for 4h at 23°C (Fig. 4).



Figure 1



Figure 2



Figure 3



Figure 4

AI was performed using a modified technique described by Arce et al. (2000). Females were immediately placed in 200 L spawning tanks. The following morning, spawning tanks were examined to determine nauplii number. Spawning success and nauplii produced per spawn were statistically analyzed using JMP version 7.

Experiment 1 was designed to compare the fertility yielded with diluted vs. undiluted sperm on 104 females. Treatment A consisted of using half sperm mass diluted technique with 20 mL of ASW (n=56), and treatment B consisted of using half sperm mass undiluted (n=48). Sperm mass was equally divided and each half was used to inseminate one different female, obtaining 1:4 male-female ratio.

Experiment 2 compares fertility yielded on three treatments: (MP) undiluted sperm mass hatching laboratory technique (n=12), (E1) undiluted sperm mass same as MP but pouring ASW on it as a humectant (n=12), and (D1) half sperm mass diluted technique with 20 mL of ASW (n=10). Obtaining two different male-female ratios 1:2 (MP/E1), and 1:4 (D1).

Results

When viewed using fluorescence microscopy with a 490-nm excitation wavelength, spermatozoa that fluoresced bright green were classified as being intact, while those stained red were classified as damaged (Fig 5). Viability/membrane integrity was approx. 97% during the first 12 h at 23°C.

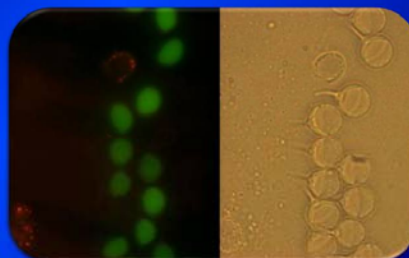


Figure 5. Left: stained sample view on fluorescence microscope, the green particles are counted as live sperm cells, the red ones are non viable. Right: same sample view on light microscope.

For Experiment 1, AI treatments were evaluated using Wilcoxon rank nonparametric test (Table 1). Treatment A yielded more nauplii number per female than treatment B. An alternative analysis approach was performed transforming nauplii number (Box-Cox) and using linear model which confirmed nonparametric results ($P < 0.001$).

Table 1. Treatment comparisons

Treatment	Nauplii Number		1Std Dev
	Median	Mean	
A	20,000	6,316.9	3002.5
B	5,000	4,158.8	3767.5

¹BoxCox transformed

For Experiment 2, we found a significant difference between treatments using Kruskal-Wallis test ($P < 0.0070$), and made a multiple comparison between treatments in order to determine which treatments differed (Table 2). Treatment MP yielded more nauplii number per female than treatment D1. Treatment E1 was not different from MP ($P > 0.05$), although there was marginal difference ($P < 0.10$). An alternative analysis approach was performed transforming nauplii number (Box-Cox) and using using linear model which confirmed nonparametric results ($P < 0.03$).

Table 2. Multiple comparisons between treatments

LSMeans Differences Tukey HSD				
a= 0.050 Q= 2.46966				
	Mean(j)-Mean(i)	LSMean(i)		
D1	Std Err Dif			
	Lower CL Dif			
	Upper CL Dif			
		0 -886.75	-2072	
E1		0 693.251	732.154	
		0 -2398.9	-3880.2	
		0 1025.34	-263.82	
		666.755	0 -1385.2	
MP		693.251	0 732.154	
		-1025.3	0 -3193.4	
		2398.85	0 422.931	
		2072	1385.24	0
	732.154	732.154	0	
	263.823	422.93	0	
	3880.17	3193.42	0	

Level

Sq Mean

MP A 3375.4926

E1 A B 1990.2488

D1 B 1303.4943

Levels not connected by same letter are significantly different.

MP-undiluted sperm mass hatching laboratory technique, E1-undiluted sperm mass same as MP but pouring ASW on it as a humectant, D1-half sperm mass diluted with ASW.

Conclusions

Different staining techniques have been successfully used to evaluate viability of *P. vannamei* sperm previously and results of this study indicate that fluorescent staining kits may be appropriate for determining sperm viability in this species.

The added volume provided by the extender improved the male to female ratio, from 1:1 of natural mating and 1:2 using the classic IA method to 1:4 using this approach, allowing us to increase the number of females to be mated to a given male.

Although hatch rate was significantly different between AI using one undiluted sperm versus half diluted one in Experiment 1; while in Experiment 2 there was a difference in favor of the routine undiluted treatment of AI currently used by Maricultura del Pacifico, all treatments yielded nauplii production, allowing an increase in the number of females mated to one male. Further development and testing of this procedure and associated cost analysis could provide the necessary information for commercialization of short-term preserved sperm.

Literature cited

Arce, S.M., Moss, S.M., Argue, B.J., 2000. Artificial insemination and spawning of pacific white shrimp *Litopenaeus vannamei*: implications for a selective breeding program. UJNR Technical Report 28, 5-7.
Leung-Trujillo, J.R., Lawrence, A.L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. *Aquaculture* 65, 363-370.

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