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## Title page

# Reproductive behavioural differences between wild-caught and pond-reared *Penaeus monodon* prawn broodstock.

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## Abstract

Ongoing problems exist with the commercial scale domestication of *Penaeus monodon*. One of the major issues, in terms of reproductive performance, is the low egg hatch rate of eggs from these captive bred prawns. The current study investigated the related issue of mating success. Time lapse video observations were conducted to compare the mating behaviour of pond-reared (domesticated) and wild-caught prawn *P. monodon* broodstock.

Mating success of the pond-reared prawns was found to be low relative to wild-caught. It was determined that both male and female prawns contributed to this low mating rate suggesting both genders were impacted negatively by the domestication process. The causative factors for the low mating success are yet to be determined, however external physical abnormalities and lack of sexual maturity did not appear to play a role. The most notable behavioural difference between wild-caught and domesticated prawns was a reduced level of pursuit behaviour by domesticated males. This, and other behavioural differences are discussed in relation to an increasing body of evidence that male prawns respond to sex pheromones produced by receptive females and that males detect these chemical signals in part, via their second antennal flagella. Accordingly we hypothesise that pond-reared (domesticated) females may have a reduced ability to produce or release sex pheromones and males, a reduced ability to detect them when compared to their wild-caught counterparts.

## 1 Introduction

A number of programs have been initiated to domesticate (closing the life cycle in captivity on a commercial scale) the black tiger shrimp (*Penaeus monodon*), an important aquaculture species (Bierne et al, 2000, Chamberlin 2003, Chung et al 2011, Coman et al 2009, Jones and Lai 2003, Macbeth *et al.* 2007, Wyban et al, 2007 a, b). Closing the life cycle of this species could relieve the industry's current dependence on wild-caught broodstock and assist development of specific pathogen free (SPF) broodstock and genetically improved culture lines. Without full domestication, diseases are commonly introduced onto farms via the wild-caught broodstock. These diseases include viral pathogens which are believed to be the major factor responsible for the dramatic worldwide decline in *P. monodon* production since 1997 (Spann and Lester 1997, Fegan 2002, Tanticharoen et al 2008). To date however, most *P. monodon* hatchery operators prefer to use wild-caught broodstock because domesticated broodstock show relatively poor reproductive performance. Reproductive criteria where domesticated broodstock are inferior include; lower spawning rates, delayed spawning, lower egg hatch rates and poorer larval survival.

Many factors have been shown to contribute to poor egg hatch rates in domesticated stock (Hansford and Marsden 1995, Wouter et al 2001) with much

attention focused on underlying egg and sperm quality issues (Coman et al 2006). Potential differences in mating behaviour between wild and domesticated stock has received relatively little attention despite being the important first step in the process of egg fertilization.

Mating in *P. monodon* and other 'closed thelycum' penaeid species, occurs at night between a post-moult female and an intermoult male (Primavera 1985). Wild-caught *P. monodon* have been shown to mate successfully in captivity when exposed to a range of tank shapes and environmental conditions (Primavera 1985, Wouter 2001). Evidence that mating has been successful includes a non-zero egg hatch rate. Where, however, zero hatch rates are recorded, and no further observations are made, it is difficult to isolate the causative agent(s) which may include sperm and egg quality and/ or failure to mate. For *P. monodon*, direct evidence of mating success can be found by visual examination of the thelycum of the moulted female to confirm the presence of spermatophores (Makinouchi and Hirata 1995). This practice is typically carried out on the morning after the female moults but this data is rarely recorded as the inspection is a stressful process for the newly moulted female. Perhaps the strongest evidence that low mating success contributes to low egg fertility in domesticated *P. monodon* is that artificial insemination (Laxminarayana and Muthu 1984) can increase egg hatch rate significantly (Kenway et al 2006), validating that, under these circumstances, egg and sperm quality are not always the causative agents.

Gender contribution to the reduced egg hatch rate in domesticated *P. monodon* was addressed in a study conducted by Coman et al (2006). After comparing hatch rates from reciprocal matings (wild-caught males or wild-caught-females are replaced with domesticated counterparts) they reported that the domesticated females, but not males, caused an increase in the number of spawnings that did not hatch. Thus from their study we know that domestication compromised female fertility, however the question remains; was it due to a reduced ability to attract males and mate, or issues related to egg quality?

In an earlier study Menasveta et al (1993) also used reciprocal matings to isolate male and female effect on fertilization rates of domesticated and wild-caught *P. monodon* and showed that both males and females contributed equally to the 45% decline in egg fertilization rate that occurred when either. Mating success, however, was not recorded in their study, so its contribution to the reduction in egg fertilization remains unknown.

Thus the current study was designed to clarify whether differences exist in mating behaviour and hence mating success, between wild-caught and domesticated broodstock and whether this may contribute significantly to reported poor hatch rate of eggs from domesticated *P. monodon*. To this end, we monitored the behaviour of pond-reared vs wild-caught *P. monodon* males and females under laboratory conditions using time lapse video recordings. Observations were recorded of the events and stages at which alteration or interruption of the mating

process occurred in the domesticated broodstock relative to the wild-caught. In addition, we reciprocally crossed wild-caught and domesticated males and females to determine the male and female contribution to any observed behavioural differences related to a decline in mating success.

## 2 Material and methods

### 2.1 Experimental prawns

Prawns used in the experiments came from two sources: (i) third generation pond-reared (domesticated) (D) prawns and (ii) wild-caught sexually-mature prawns (W) collected from near shore waters, North Queensland, Australia. The D stock consisted of 30 males and 30 females that were 14 months old. Individuals were harvested from a 200m<sup>2</sup> plastic lined pond at the Bribie Island Research Centre (BIARC) located in southern Queensland. Prawns were reared at an average density of 4 m<sup>2</sup> and were fed twice daily on a diet consisting of high protein pellets (Higashimaru- *Marsupenaeus japonicus* diet) with a twice weekly supplement of fresh-frozen mullet and squid. For the W treatment group, 25 females and 20 males of unknown age were captured from fishing grounds off Cairns in north Queensland and air freighted to BIARC in southern Queensland. The founding stock for the domesticated line came originally from the same spawning grounds as the W stock.

The average size for a pond-reared male and female prawn was 78.4±1.2 grams and 94.6±2.0 grams, respectively, and 84.2±1.8 grams and 105.6±0.9 grams, respectively for wild-caught individuals.

### 2.2 Holding facilities

Prior to transfer to holding tanks, prawns were physically examined for any abnormalities (including in external genitalia and antennule damage). Prawns were then eye tagged for individual identification, weighed and moult staged assessed according to Promwikorn *et al.* (2004). After any prawns with damaged antennae were rejected, remaining individuals were transferred to a holding tank and held for a seven day acclimation period at a density of 2 per m<sup>2</sup>. After acclimation, pre-moult females were transferred to a small (3m x 2m) holding tank (Tank A) in a temperature and light controlled room. Approximately six inter-moult males (W and D lines) were also transferred to Tank A. Water temperature in holding tanks was maintained at 28°C with a 150% water exchange conducted daily and individuals fed a diet of fresh frozen squid or mussel on alternative days to excess twice daily.

### 2.3 Observation tanks

Three time lapse video surveillance cameras (Sony) were mounted above three circular observation tanks (diameter of 1.5 m 1.2 m depth). Each afternoon tanks were filled with filtered (20 $\mu$ m), preheated seawater (28°C) to a depth of 1 metre. No water exchange occurred and the room was heated to 28°C to maintain water temperature. A single air stone in each tank released a fine stream of bubbles that maintained O<sub>2</sub> levels at 8 mg/L without disturbing the water surface or distorting the video image. Lighting was supplied by red bulbs positioned 1 metre above each tank. Observation tanks were cleaned and refilled daily.

### 2.4 Observations

At 18:00 hrs the 3 pre-moult females most likely to moult were transferred from Tank A to the 3 observation tanks with two inter-moult males (see Table 1) and one inter-moult female (of the same origin as the pre-moult female) for overnight video surveillance. The following morning males and inter-moult females were returned to their respective tanks. Any moulted female from the observation tank was returned to the acclimation tank. If a female moulted in the observation tank, the video cassette was given a confidential coding, to enable subsequent viewing and assessment of behavioural criteria to be made without prior knowledge of the origin (W or D) of the female.

Table 1

### 2.5 Behaviour classification

Various pre-mating, mating and post-mating male and female behaviours were recorded from the video films and a brief description of these are given in Table 2. As no interactions were observed between the intermoult female and other prawns in the viewing tank, her data are not included.

If two males pursued the moulted female, only the behaviour of most active male (most time moving) was recorded.

Distances of males from the female were estimated in terms of prawn body lengths with one body length being 20cm.

'Male moving' was defined as the percentage of time (40 minute post moult), the prawn was not stationery. 'Stationery' was when the outline of the carapace remained unmoved for more than 5 seconds. The stationery time was then totalled and deducted from the 40 minutes to give the 'time moving', which was then converted to a percentage.

### 2.6 Statistical analysis

Whether continuous variables (e.g. percent time under female) varied with male or female line (W or D) was assessed using two way ANOVA (model of 'female source', 'male source').

Whether binary variables (eg mating or not) varied with origin of male or female line (W or D) was assessed using analysis of contingency tables and a chi-square test. Restricting the analysis to data where all of a single sex belonged to the same line (i.e. W or D) permitted us to assess the effect of female line after controlling for the male line (W or D).

Whether the predictor variables (e.g. percent time under female) could predict successful mating or not, was assessed using binomial logistic regression. To identify the most important predictor/s of mating success we used forward stepwise logistic regression procedures.

All statistical analyses were performed in SPSS. Significant level  $<0.05$ .

Proportions were normalized using arc sine square root transformations, and where appropriate, data were normalized by natural log transformation.

### 3 Results

Table 1

Table 2

Matings were observed only involving pairings where the female originated from the wild (row 2, Table 2). Contingency table results show that there was a significant effect of female line on mating success (pooling all males,  $\chi^2_1 = 7.446^{**}$ ) but not for male line (after all females were pooled). The effect of female line was significant when only wild (W) males were considered ( $\chi^2_1 = 5.538^*$ ), but not for domesticated (D) males.

Moulted females were pursued more vigorously by W line males than D line males. Specifically, W line males spent more time “under” newly moulted females than did D line males ( $F_{1, 27} = 15.487^{***}$ .) Similarly, W line females more often had males “under” them (two way ANOVA  $F_{1, 27} = 16.804^{***}$ ) than did D line females (row 3).

This pattern was also evident at a distance of 20-40 cm from (just behind) the female. W line females more often had males in 20-40 cm proximity than did D line females ( $F_{1, 27} = 6.192^*$ , row 3, Table 2), and W line males spent more time in close proximity to females than did D line males.

A significant difference was detected between W and D line individuals, for the time of night at which moulting occurred. On average, D females moulted 7:48±0.44 hours after they were transferred to the observation tank. This was a significantly longer waiting period than for W females (5:16 ±0.30) ( $F_{1, 28} = 8.054$ ,  $P < 0.01$ ) (Figure 1). In addition, as a group, D females had a more protracted moult period (duration 10hrs), than the W females (duration 6 hrs) (Figure 1) with the latest D moult occurring at 06:30, compared to 02:15 for the W line. Time taken for the female to exit the moult was not significantly different for D and W females (Table 2).

#### Figure 1

Overall, the time males spent moving was significantly higher when W line females, rather than D line females, were present in the observation tank ( $F_{1, 27} = 18.514^{***}$ ) (Table 2). The time males spent moving was also higher for W rather than D line males ( $F_{1, 27} = 7.146^*$ ).

Males were aggressive (pushed or chased each other) more often when chasing a W line moulted female than a D line female (all males pooled,  $\chi^2_1 = 5.011^*$ ). The number of aggressive encounters recorded however, was not influenced by male line (W or D)

Males also cleaned their antennae more often when exposed to a D line moulted female than when exposed to a W line moulted female ( $F_{1, 27} = 4.72^*$ ), but male line (W or D) was not associated with this behaviour.

Males of both lines showed a higher incidence of contacting (touching) the discarded shell of a female from the D line than that of the W line (pooling all males,  $\chi^2_1 = 10.444^{***}$ ).

No statistically significant interactions were detected.

#### 4. Discussion

The observations made in this study confirm that lack of natural in-tank matings of domesticated *Penaeus monodon* can contribute to the low egg hatch rate of these stocks (Coman *et al* 2009, Macbeth *et al.* 2007). While mating occurred in 60% of the wild-caught pairings, no matings occurred for pairings of pond-reared male and female prawns.

By reciprocally crossing wild-caught and domesticated (pond-reared) broodstock, it was also shown that both male and female prawns contributed to the low mating success of domesticated prawns. A male effect has previously been reported by Makinouchi and Hirata (1995) who determined that spermatophore implantation in wild-caught female *P. monodon* (a direct indicator of mating success), decreased from 66.7% to 32.0% when wild-caught males were



replaced by domesticated males. Their study did not, however, evaluate the effect of domesticated females which, in the current study, were shown to play a greater role than the males in the reduction of mating success. Specifically, when wild-caught males were replaced by domesticated males and paired with a wild-caught female, mating occurred in 28% of observations while if wild-caught females were replaced by domesticated females and paired with a wild-caught male, no mating occurred.

The observed effect of female origin (wild-caught versus domesticated females) on mating success was not directly evident in female behaviour. This is because females play a largely passive role in the mating process; they moult and swim regardless of male pursuit. The effect of female origin was however clearly evident in the males behavioural response to the female, most notably pursuit vigour. However, there was one important observable difference in female behaviour due to origin and that was the time when the females moulted. On average, domesticated females moulted later in the night (02:30) than wild-caught females (23:30). Also the range of times over which individuals moulted was greater for domesticated females.

One possible explanation for the delayed and protracted moulting period of domesticated females, is that the domestication process has somehow altered the physiological state of the prawns and thereby, their response to diurnal cues. This compromised or altered physiological condition of the domesticated prawns may also be responsible for what we hypothesise is a 'reduction in pheromone production or detection in females and males respectively, leading to reduced mating success.

Observations made in the current study confirm that *P. monodon* employs the 'pure search' mating strategy that is common to many shrimp and prawn species (Bauer and Caskey 2006). When matings did occur between domesticated males and wild-caught females in the current study, the observed sequence of events did not differ from that of wild-caught pairings in the current study and were as described by Primavera (1979) for wild-caught *P. monodon*. However, domesticated males rarely entered the first phase of the mating process; pursuit of female. If pursuit did commence, the intensity of pursuit (as measured by the percentage of 'pursuit time' the male spent under the female) was lower than for wild-caught males. In observations where domesticated males did pursue domesticated females, in no case did this advance to the phase of copulation (rotation, embrace and spermatophore transfer (Primavera 1979)).

It is difficult to isolate factors responsible for this noted reduction in 'pursuit vigour' of domesticated males. Rearing environment, nutritional status and genetics, amongst other things, would each play a role (Benzie 2008, Harrison 1990, Wilder et al 2010, Wouters et al 2001). We can however eliminate two potential factors; physical deformity and lack of sexual maturity. Firstly, prawns with abnormal phenotypic features were excluded from the study, reducing the likelihood that physical disability reduced mating success. Secondly, we have

supporting evidence that prawns were sexually mature, thus reducing the possibility that the domesticated prawns were too young or small to mate. Specifically, domesticated prawns from the same cohort, size range and geographical origin, were shown to be mature in a separate spawning trial where females were artificially inseminated and fertilised eggs were produced (data not shown). Also, while both domesticated male and female prawns were on average smaller ( $78.4 \pm 1.2$  and  $94.6 \pm 2.0$  respectively) than their wild-caught counterparts ( $84.2 \pm 1.8$  and  $105.6 \pm 0.9$  respectively), other studies have shown that domesticated *P. monodon* of this size are sexually mature (Coman et al 2006). Further, all male *P. monodon* used in the current study were above 70g, which was shown by Jiang et al (2009), to be the average minimum weight for males to possess viable sperm, signifying sexual maturity.

Thus, while results of the current study showed that arousal level and subsequent pursuit vigour of domesticated males was low compared to wild-caught males, the reason for this is yet to be determined. For *P. monodon* and other 'closed thelycum' prawn species, male pursuit of the female is triggered by the female moult (Primavera 1979). Studies on other species of crustaceans have shown that pheromones (Wyatt 2010) act as evolved, physiological cues to direct specific mating behaviours (Rittschof and Cohen 2004, Diaz and Thiel 2004). Observations support the hypothesis that soluble, distance pheromones may direct males to receptive females and then stimulate copulation (Zhang et al 2010). The number, structure and mode of action of prawn sex pheromones however, are yet to be confirmed. If pheromones are the means by which females communicate their readiness to mate, then domesticated females may release a lower concentration of pheromones or the chemical structure may be modified and therefore not trigger the normal male response. Also, the males contribution to the low mating rate may be due to a reduced ability to detect pheromones when compared wild-caught males.

Physiological studies on crustaceans indicate that antennae and antennules are major chemosensory organs in many crustaceans (Kamio et al 2005) and could be directly involved in male detection of receptive females (Bertin and Cézilly 2003, Bauer and Caskey, 2009). Observations made in the current study also suggest that the antennae and antennules play a role in the mating process for *P. monodon*. For example, after females moult, males were observed cleaning their antennules (passing the full length of the antennules past their mouth area) and opening their antennule scales. This action is thought to concentrate and thereby aid males in the detection of the water soluble sex pheromones released by moulted females. There was a trend towards this behaviour being more frequent, when the female originated from domesticated stock indicating that water born pheromone signals were detected but not sufficient for males to track females.

In addition to the possibility that water-born pheromones help direct the mating process in *P. monodon*, chemo-tactile cues (non-soluble chemicals detected by touch) could also be playing a role. Specifically, contact pheromones (notably,

insoluble coatings on the surface of the female moult) may also be used by males to identify and track receptive females (Baeuer et al 2009, Caskey and Bauer 2005, Caskey et al 2009). Males in the current study occasionally touched or pulled at the discarded moults of females (data presented as 'male on moult'). As with the pursuit of females, this behaviour was male specific. Contact with the female moult was always accompanied by the male cleaning his antennules (data presented as 'clean') and moving antennal scales, after which males usually commenced or continued their pursuit of the moulted female. Interestingly, 'male on female moult' was shown statistically to be one of the best predictors of female line (W or D). Specifically it was observed more frequently when the female was domesticated than if she was wild-caught. As with the male 'cleaning' behaviour, this behaviour may be part of the males attempt to concentrate the chemical(s) and thereby strengthen the weak pheromone signal from the female and aid in her identification.

## Conclusion

Results of this study confirm reduced mating rates can contribute to poor egg hatch rates found for domesticated *Penaeus monodon*. The observed poor mating success of these prawns was due to both genders. Domesticated males showed a lack of vigour in their pursuit of moulted (receptive) females compared with wild-caught males and domesticated females had a reduced ability to stimulate males.

The decline in reproductive success is discussed with regards to published evidence of pheromones playing a regulatory role in crustacean reproductive behaviour. Specifically, evidence that males detect pheromones released by the female during moulting and these water soluble chemicals drive male mating behaviour. We then hypothesise that this signalling process may be compromised in domesticated prawns reducing mating success. Domesticated females may produce less or ineffective pheromones, and domesticated males may have a reduced ability to detect or process the chemicals, when compared to their wild-caught counterparts. To improve *P. monodon* mating success in captive environments, and to relieve the industry of the need to artificially inseminate domesticated prawns, factors that control or influence mating processes require further investigation.

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Table 1: Pairings of male and female prawns placed in observation tank for videoing.

Origin of prawn	Females (1 pre-moult and 1 inter-moult)	Males (2 inter-moult)
	W	W
	W	D
	D	D
	D	W

Table 2 Mating Behaviour

For gender of	Behaviour	D♀ D♂ n=6	D♀ W♂ n=8	W♀ D♂ n=7	W♀ W♂ n=10
		♂			
♂	Matings observed. Mating is defined as males wrapping around the female and showing rapid muscle contractions. Verified by visual examination of the females thelycum the following morning; swelling and tissue protrusion. Recorded as "yes" / "no" for each female and tabulated as a percent of "yes" out of each n value (where n = number of trials).	0% <sup>a</sup>	0% <sup>a</sup>	28% <sup>a,b</sup>	50% <sup>b</sup>
♂	Time under ♀. Defined as the percent of time a male spent under a female++.	1.67 ±1.67 <sup>a</sup>	12.50 ±4.73 <sup>a</sup>	18.57 ±8.36 <sup>a</sup>	60.50 ±7.32 <sup>b</sup>
♂	Time 20-40cm from ♀. Time as a percent 20-40cm from female ++	1.67 ±1.05 <sup>a</sup>	5.00 ±2.11 <sup>a,b</sup>	5.00 ±1.89 <sup>a,b</sup>	10.00 ±1.49 <sup>b</sup>
♂	Time moving. Percentage of time moving ++	32.50 ±6.16 <sup>a</sup>	43.75 ±6.47 <sup>a</sup>	57.86 ±9.57 <sup>a</sup>	85.50 ±7.54 <sup>b</sup>
♂	Male fighting. Number of trials where males jostled for position and or chased each other before returning to the female. Tabulated as a percentage. of each n value (where n = number of trials).	0% <sup>a</sup>	25% <sup>a,b</sup>	43% <sup>a,b</sup>	60% <sup>b</sup>
♂	Cleaning. Number of times a male 'cleaned' its	4.67	8.13	3.29	2.60



	antennae(one or both) within one hour post female moult.	$\pm 1.89^a$	$\pm 2.32^a$	$\pm 1.09^a$	$\pm 0.60^a$
♂	♂ on ♀ moult. Whether a male, touched the females discarded moult/shell ++. Recorded as "yes" / "no" and tabulated as a percent of "yes" out of each n value (where n = number of trials).	100% <sup>a</sup>	100% <sup>a</sup>	28% <sup>b</sup>	60% <sup>b</sup>
♀	Time (in seconds) to exit moult (shell).	4.00 $\pm 0.26^a$	3.88 $\pm 0.23^a$	4.00 $\pm 0.22^a$	3.90 $\pm 0.23^a$

Values for mating combinations across a row with the same superscript letter are not significantly different from each other. ++ During a 40 minute post moult time interval or until mating occurred.

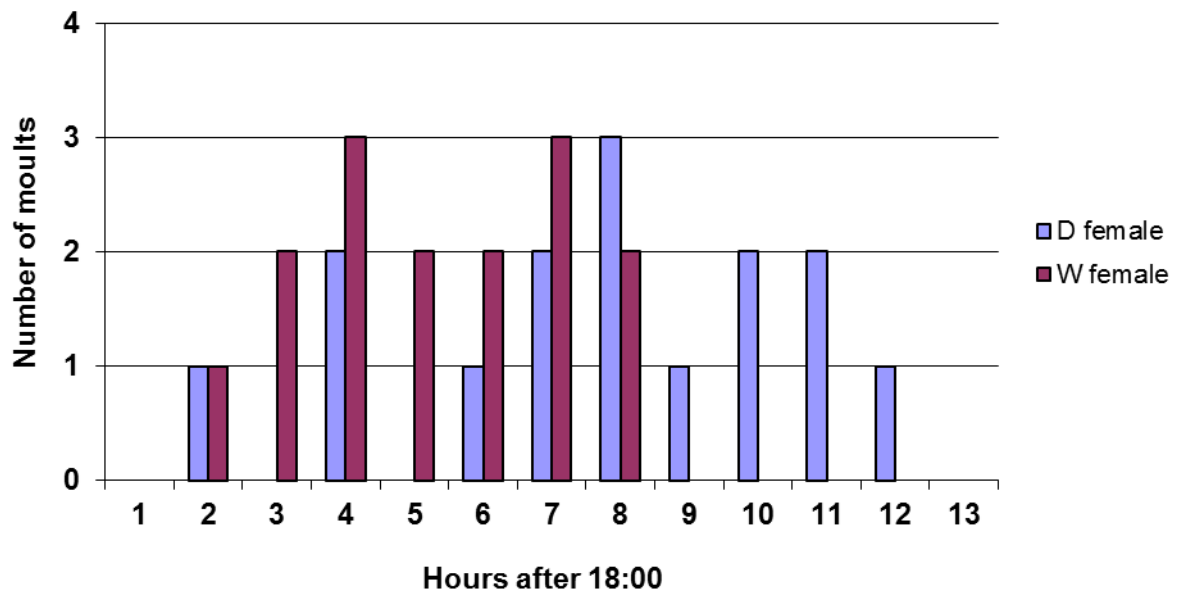


Figure 1 The number of moults occurring for W (n=15) and D (n=15) females in each 60 minute time interval after transfer at 18:00.

**Highlights**

- We recorded observations of mating behaviour in wild-caught and domesticated *Penaeus monodon*.
- Mating success of the domesticated prawns was low relative to wild-caught.
- Both genders contributed to the low mating rate of domesticated prawns.
- Domesticated males showed a lack of vigour in their pursuit of moulted (receptive) females.
- Domesticated females had a reduced ability to stimulate males.

ACCEPTED MANUSCRIPT