Towards all female P. monodon populations using endocrine manipulations

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Principal Investigator: Prof. Abigail Elizur

Background

Australian prawn aquaculture production is based predominantly on *P. monodon* farming. The Australian prawn industry is challenged by the lower priced overseas imports, which are mainly smaller sized prawns. Larger sized prawns attract premium prize in the local market. *P. monodon* females grow significantly larger than males, and hence, a technology to develop all-female monosex populations would offer competitive advantage at a local as well as international market to the Australian seafood industry.

This project followed upon results achieved in previous three years of research funded by QICARP (Queensland-Israel Collaborative Agriculture Research Program). During this three year period, our research led to the finding of the androgenic gland in *P. monodon* and the gene *PmIAG,* which through its product *PmIAGh,* is most likely to be the candidate responsible for male differentiation. Subsequently, external phenotypic changes were achieved upon single implantation of the androgenic gland into juvenile female prawns, though no internal changes were affected. Hence it was proposed within this project that repeated administration of the AG into younger juveniles could lead to the desired full sex reversal i.e. ZW neo-males. When these ZW neo-males are crossed with ZW females, 25 % of the resultant progeny will be of WW female genotype. These WW females will form the basis for the production of all female population when mated with wild ZZ males. In sequel to encouraging results achieved with regard to the masculinising effects of the androgenic gland (AG) in *P. monodon*, a strategy to conduct multiple implantation/injection plans was devised to maintain a sustained dosage of the AG hormone. This approach was reasoned to be necessary to influence a larger time-

frame during which the larvae could be sexually labile. Concurrently, improved delivery methods were devised to target young larvae.

Alternatively, oral delivery of methyl testosterone (MT) by way of enriched diets was carried out to find its effects on masculinisation of *P.monodon* larvae. Testosterone levels have been reported in crustaceans. It is not known whether crustaceans are capable of synthesizing testosterone endogenously or accumulate through exogenous sources (Vogt, 2007). Studies on the influence of testosterone in crustaceans indicate that it has wide ranging effects on reproductive organs, sex ratios and sex reversal. In *Parapenaeopsis hardwickii*, administration of exogenous testosterone resulted in enhanced spermatogenesis (Nagabhushanam and Kulkarni, 1981). Macrobrachium rosenbergii larvae fed with MT enriched artemia nauplii resulted in altered sex ratios in favour of males (Baghel et al., 2004). These supporting evidences were the basis for extending our investigation into the development of neo-males as a preliminary step towards production of all female population in *P. monodon*.

Initial Project Objectives:

1. Optimise procedure for neo-male creation using androgenic gland implantations and MT treatments.
2. Cross neo-males with females to establish viability and fertility of WW prawns.
3. Grow WW P. monodon females on farm and research provider facilities to reach sexual maturity.
4. To cross WW P. monodon females with wild males (both on farm and at the research provider facility) and obtain viable all-female offspring.
5. To assess the reproductive performance of WW females compared to normal WZ females.
6. Assess production performance of all-female offspring compared with mixed population (on farm).

Methodology:

Androgenic gland implantation

Multiple androgenic gland extract injections and multiple androgenic gland implantations were conducted on young *P.monodon* juveniles as a strategy to have a sustained presence of androgenic gland hormone over a longer timeframe during which the prawn juveniles were considered to be sexually labile. In all, 2 injections and 3 implantations were conducted starting with 400 juveniles. Alternatively, methyl testosterone, was orally administered through enrich diets at three dosage levels of 50, 100 and 150 mg/Kg of enriched diets.

400 Post larvae of average 245mg were injected with hypertrophied AG extract (Table I). Two AGs were homogenised with 80ul crustacean saline. 2 ul of the homogenate were injected into each animal. This was repeated 2 weeks later on 317 surviving prawns with an increase in dosage. A month after the start of the trial, 137 prawns survived, which were implanted with 1/8 of an AG. This was repeated after 2 weeks, on 133 surviving prawns at the dosage of 1/6th of AG/prawn. 37 prawns were subjected to a third round of implantation 2 weeks later at the dosage of 1/4th AG/prawn and later released and maintained in a covered heated pond (N1). Untreated, control prawns were also released in to N1.

Table I. Injection and implantation of androgenic gland into *P. monodon* juveniles.

Injection/ implantation	Weight (g)	Stage	Number of prawns implanted	Survival (%)	Prawns per gland	Approx. Dosage of gland per prawn (mg)
Injection	0.245	PI 38	400	100	20	0.4
Injection	0.500	PI 44	317	79.25	12	0.66
Implantation	1.380	PI 56	137	34.25	8	1.00
Implantation	2.49	PI 70	133	33.25	6	1.33
In addition, a third implantation on 37 surviving prawns was conducted before releasing in to covered heated pond (N1). 50 prawns injected concurrently with crustacean physiological saline served as controls. The trial was conducted over a period of 90 days.						

Oral administration of 17-α methyl testosterone (MT)

Post larvae 20 (20 days after metamorphosis) were fed with a combination of MT (Sigma-Aldrich Ltd, Australia) enriched live artemia nauplii (Inve Technologies nv, Belgium) and MT enriched post larval particulate diet (Inve Technologies nv, Belgium and Charoen Pokphand Group, Thailand) for a period of 10 days and thereafter the post larvae were fed with MT enriched particulate diets for a further period of 78 days, thus totalling 88 days of treatment.

Three concentrations were chosen for treatment of the post larvae: 50 mg/Kg, 100 mg/Kg, 150 mg/Kg and Control with no MT. These were conducted in duplicates thus totalling 8 tanks of 500 litre round tanks with parabolic bottom. In each tank three hundred post larvae 20 (PL20) were introduced. These were later transferred to 4x 6000 litre parabolic tanks when the juveniles achieved benthic character.

Transcriptome analysis

Transcriptome analysis of the androgenic gland was carried out. We obtained approximately 30,000 reads following submission to the AGRF genome sequencing facility. Analysis is under progress. The sequence information would elucidate the transcriptional complexity of the gland and reveal potential hormones that could have a significant role in masculinisation.

Achievements:

Results of AG manipulations:

A total of 37 prawns were released into a covered heated pond along with 10 control males and control females. The prawns were fed twice daily with commercial prawn pellets and were reared for 8 months. Water quality was monitored and maintained at Temperature: 26 to 30 degree Celsius; Salinity of 34 to 36 ppt; pH of 7.8 to 8.2 and a dissolved oxygen levels of above 4 ppm. Recovery of animals at harvest was very poor at 3 males and only 1 female. After duly clipping the uropods, the males were analysed with sex markers (Staelens et al., 2008) for their genotypic sex.

Repeated Implantation of the AG into prawn juveniles resulted in phenotypic transformation which included the appearance of appendix masculine and enlarged petasma (Fig. 1). The transformation however did not result in sex change or the creation of neo males (Fig. 2).

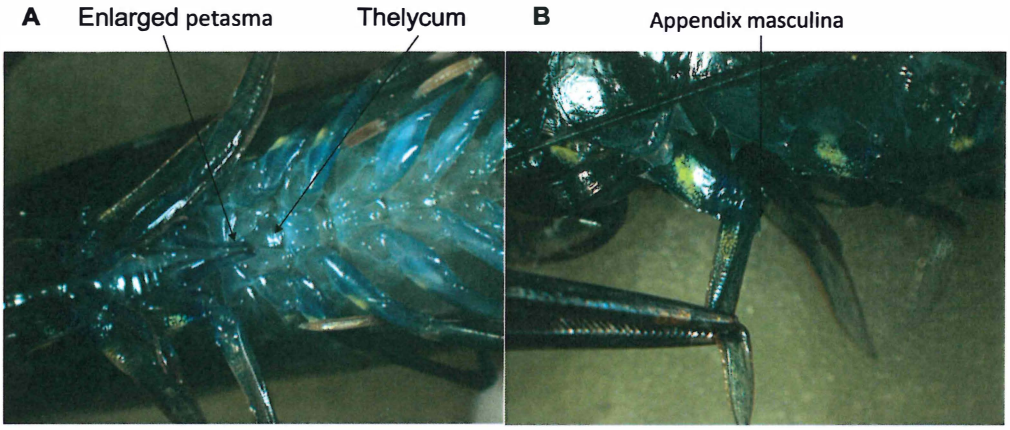

Figure 1. Transforming ZW female (34.0 g) showing phenotypic male characteristics following AG implantation. A: Enlarged petasma and B: Appendix masculine, both of which are characteristic of males.

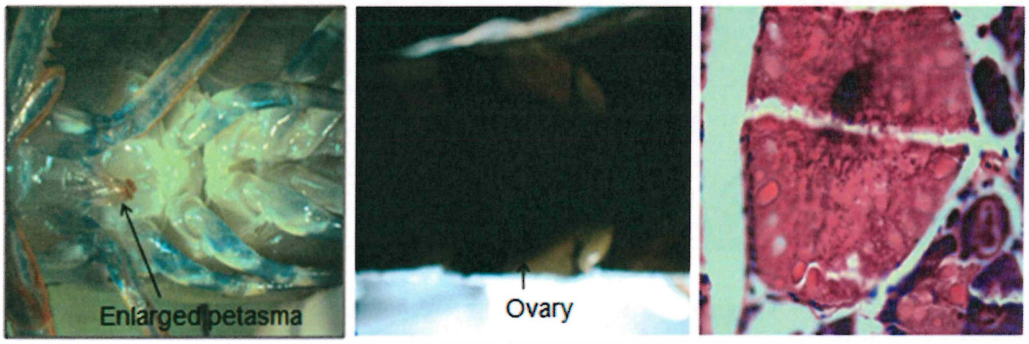

Figure 2. A masculinised ZW female (46.0 g). **A:** Showing enlarged petasma **B:** Showing developed ovary beneath the 1st abdominal segment* and **C:** Ovarian cell indicative of active vitellogenesis.

**photo of ovary taken from a live prawn by focussing light through the abdomen.*

Results of MT treatment trial:

At all 3 concentrations of 50, 100 and 150 mg/Kg of orally administered methyl testosterone enriched feed, no sex reversal characteristics were noticed. 20 males

from each concentration were tested with sex specific markers to confirm that their genotypic sex is no different from observed phenotype. Several prawns were observed with severe deformities at 150mg/Kg treated groups including malformed thelycum, and missing legs around the reproductive parts (Fig. 3).

Table II: Sex ratios and average body weight in grams of prawns exposed to MT treatments.

Treatment	50mg/kg	100mg/kg	150mg/kg	Control
	N	N	N	N
Males	60	51	22	22
Females	44	43	30	23
M to F ratio	1.36	1.18	0.59	0.95

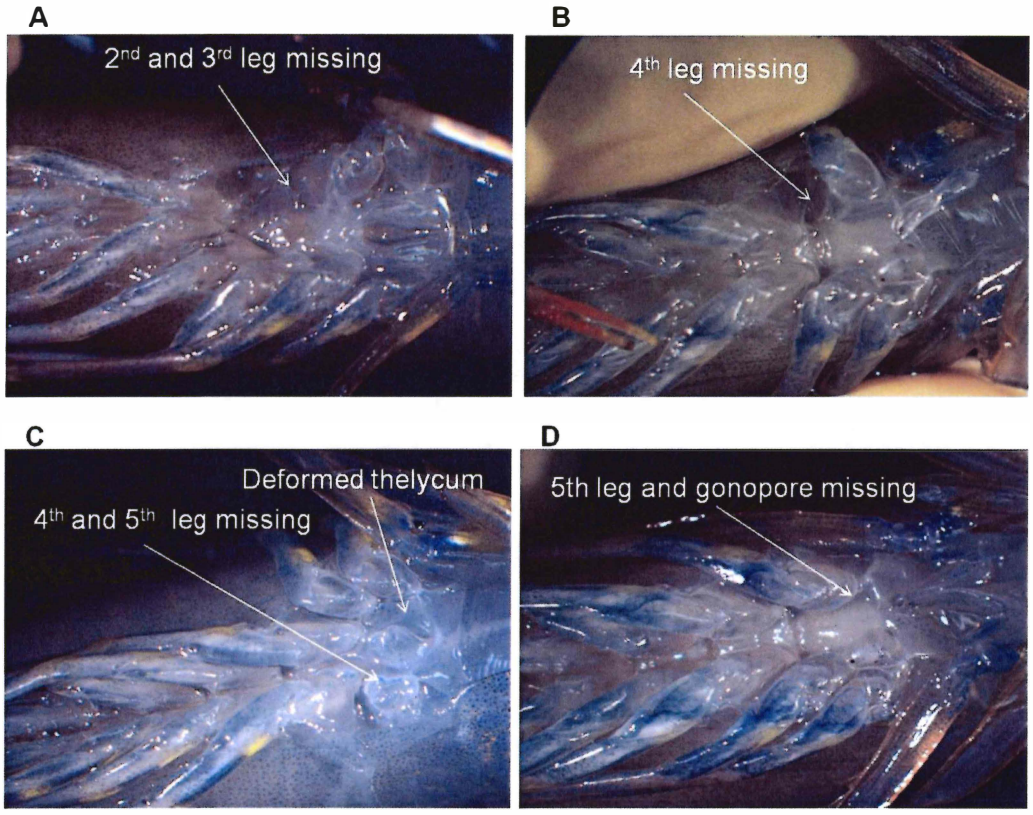

Figure 3. Abnormalities observed among the various MT treatment groups (~4.5 g and ~6.0 g). **A**: Left 2nd and 3rd leg missing in 50mg/Kg treated group female. **B**: Left 4th leg missing in 100mg/Kg treated group male. **C**: Right 4th and 5th leg missing and

deformed thelycum in the 100mg/KG treated group female. **D**: Left 5th leg along with the gonopore missing in the 150mg/Kg treated male.

Bioinformatics analysis:

Extensive bioinformatics of the complete coding sequence of PmIAG reveals it to pre-pro-peptide of 176 amino acids encoded by 531 bases with a predicted mass of 19.85 kDa. It has a structural similarity to insulin-like family of proteins with a signal peptide at its N-terminus (33 aa) and B and A chains (45 aa and 31 aa respectively) connected by a C-peptide. The mature peptide comprising B and A chains has a predicted mass of 8.6 kDa.

To further understand the cellular and molecular processes underlying the secretory activity of the AG and in order to find novel genes expressed in the AG, transcriptome analysis was conducted on sequences obtained on a Roche 454 Next Generation Sequencing platform. While beyond the scope of this project, this platform will enable the identification of the types of proteins present in the AG and would be an important tool in understanding various factors associated with the gland and their biological roles.

Paper entitled "**Isolation and characterization of the complete cDNA sequence encoding a putative insulin-like peptide from the androgenic gland of *Penaeus monodon*"** has been published in journal Aquaculture.

With reference to the first objective, the trials using multiple injection / implantation of androgenic gland to achieve neo-males resulted only in external signs of masculinisation. Methyltestosterone treatment did not influence any masculinisation. Hence, downstream objectives could not be addressed. Since the project did not meet the agreed go-no go point, as mentioned in the project proposal, it was prematurely terminated.

Future research

There are two possible explanations for the fact that andrectomy and implantation trials led to only partial feminisation and masculinisation features respectively. It is possible that sexual differentiation in penaeids occurs much earlier than the times tested in this study, beyond which they reach "a point of no return". **Hence intervention studies aimed at earlier stages of sexual differentiation should be carried out to try and obtain full sex reversal**. It is also possible that internal sexual differentiation precedes that of AG organogenesis, in which case it is possible that alternate mechanisms, independent of AG, are involved in primary sexual differentiation in *P. monodon*. This calls for investigation involving differential expression of genes to isolate factors other than those associated with AG and development of suitable bioassays to determine their roles.

Implantation trials in this study at early post-larvae stages were constrained due to difficulties posed in implanting AG tissue into small sized post-larvae and sensitivity of *P. monodon* juveniles to handling. Development of recombinant *Pm-IAG* or extraction of the AG proteins will enable delivery via microinjection and hence aid in targeting younger stages. Further, the functional role of *Pm-IAG* in the sex differentiation pathway needs to be clarified. RNAi induced suppression of its expression in macrobracuim has resulted in complete sex reversal from male to female, however it remains to be confirmed as to whether it is involved in the primary sexual differentiation pathway or restricted to external secondary sexual characters in *P. monodon*.

The present study set out to evaluate the functional role of the AG in sexual differentiation with the main intent of generating all-female populations. Given the commercial importance of penaeid prawns, the fundamental understanding of the AG and its role in sexual differentiation is essential to the development of all-female monosex culture technologies, and this study provides the foundation for the use of AG in any further biotechnological applications.

In this study, treatment with MT did not result in sex reversal of *P. monodon*, unlike similar studies in other crustaceans. MT had a negative impact and resulted in

low levels of abnormalities. The results of the present study reflect the limitations of vertebrate steroids on influencing sexual differentiation in crustaceans.

Future recommendations:

Better characterisation of the timing of AG expression, as well as the full suite of AG specific genes are a key to the knowledge of when the gland can be used to manipulate sexual differentiation. Once this information is available, the creation of neomales can be revisited for the development of all-female monosex populations.

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