

The development of biological tagging techniques for penaeid prawns

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Non-technical summary

The objective of this project was to develop novel biological tags for penaeid prawns. The impetus for this research was the growing interest in Australia in the potential for stock-enhancement of penaeid fisheries with hatchery reared juveniles. In any stock-enhancement program some means of differentiating between introduced and wild prawns is needed to monitor the effectiveness of the program. Many different types of tags have been used in fisheries, but none are suitable for penaeid reseeded. For prawns, the tags would ideally be: able to mark individuals at all life history stages; unique to the local population; inexpensive and quick to apply and detect; either transmissible or non-transmissible to subsequent generations; and harmless to both the prawn and consumer (Rothlisberg and Preston 1992). This project examined whether novel chemical and genetic tags could meet these criteria and hence provide a means of monitoring the success of prawn stock-enhancement programs.

The results of the study showed that novel chemical and genetic techniques could be effectively used to tag prawns. Neither type of tag meet all the desired criteria but each would be well suited for different purposes in stock-enhancement trials. Chemical element tags would provide a cost-effective means of monitoring the fate of small prawns during the first few weeks after their release in pilot-scale stock-enhancement trials. If the pilot trials were successful, genetic tags could then be used in subsequent full-scale releases of permanently identified prawns. Genetic tags would also be required to monitor and maintain the genetic diversity of the enhanced populations. The main results of the study were:

Chemical elements

- Trace element composition and concentrations were highly variable within and between wild prawn tissue types and between seasons, locations, diets and sediments
- Among the trace elements that are considered essential for crustaceans, arsenic and selenium both accumulate in the abdominal muscle of juvenile prawns
- Elevated levels of a rare, stable selenium isotope (^{74}Se) can be introduced via pelleted feeds and accumulated in the abdominal muscle of juvenile prawns
- Rare isotopes of nitrogen (^{15}N) and carbon (^{13}C) which can also be introduced via pelleted feeds and accumulated in the abdominal muscle of juvenile prawns

- Introduced (^{15}N), (^{13}C) and (^{74}Se) isotopic signatures of released juvenile prawns would be distinguishable from those of natural recruits for a period of 4-5 weeks.

Genetic markers

- Foreign DNA was successfully introduced into prawn embryos via microinjection and electroporation
- Transient expression of reporter genes was induced in prawn embryos under the control of the fruit fly hsp70 and hsp82 heat-shock promoters
- Polymerase chain reaction screening of genomic DNA for *hobo*-like transposable elements gave positive results for *Penaeus esculentus*
- A provisional Australian patent on the use transposable elements as gene vectors in crustaceans has been filed (PN4593)
- Preliminary characterisation of microsatellite DNA markers in *P. esculentus* and mitochondrial DNA markers in *P. japonicus* showed that these natural genetic markers have considerable potential as tags for prawn stock-enhancement programs

Apart for the successful development of novel biological tags for penaeid prawns this project has produced important benefits for the Australian prawn farming and fishing industries. The need to maintain a supply of embryos for the gene transfer experiments resulted in the first successful captive breeding of Australian Kuruma prawns (*P. japonicus*). The captive-bred stocks were subsequently used to produce several generations of commercially farmed prawns (Preston *et al.* ^ain press). The techniques used for maintaining broodstock have also permitted the assessment of heritability for growth under controlled laboratory conditions (Hetzl *et al.* in press). Microsatellite DNA markers, similar to the *P. esculentus* markers isolated in this project, subsequently proved to be effective for determining the paternity of prawns in laboratory selective breeding trials for *P. japonicus* (Moore *et al.* in press). These advances in domestication and tagging techniques mean that it is now possible to produce and monitor genetically tagged prawns in stock-enhancement programs. It is now also possible to commence the development of selective breeding in farmed *P. japonicus*.

In this project we developed techniques for the introduction of DNA into prawn embryos (Preston *et al.* ^bin press) and transient expression of reporter genes in prawns. We also demonstrated that there are genetic elements in the prawn genome with significant homologies to transposable elements in other species. A provisional patent on the use transposable elements as gene vectors in crustaceans has been filed (PN4593). Although stable genetic transformation of prawns is yet to be achieved, we have made significant advances in the enabling technology. Ultimately, this technology could be used to

provide a benign genetic tag. However, the most significant application of this technology to penaeid prawns could be in genetically engineering traits such as improved growth or resistance viral diseases.

Background and Need

In fisheries research the ability to tag or otherwise identify individuals is of fundamental importance in studies of natural populations. Fisheries scientists have used physical and chemical tags with varying degrees of success in studying population size, growth, migration and mortality. However, no physical and chemical tags have yet been developed that can entirely overcome the effect of the tag on the animal and the likely differential survival between tagged and untagged populations. There is a need for tags that are naturally occurring or biological in origin, and where the tag is totally benign.

Penaeid prawns, like most crustaceans, are particularly difficult to mark or tag. Frequent shedding of the exoskeleton precludes the use of the microchemistry of skeletal parts, other than for short periods. Some attempts have been made to use parasites as biological markers (Owens and Glazebrook 1985) but the parasites can affect the growth and migration of the host (Somers 1991). Prawns have a dispersive larval phase that ensures a degree of genetic mixing between regions thus limiting the use of natural genetic variants as unique tags.

Physical tags, inserted into adult prawns, have been widely used in studying the population dynamics of prawns in Australia, but result in tag-induced mortality (Penn 1981). Thus, the development of cost-effective, unharmful, biological tags would be a major breakthrough in our ability to study natural population dynamics.

Biological tags applied to prawn post-larvae or juveniles would also have great potential in the evaluation of re-stocking programs that have suffered from the lack of an effective tag (Farmer & Al-Attar 1981). Such programs have been carried out for many years in countries such as Japan (Shigueno 1974) and China (Liu *et al.* 1991) but have been widely criticised for being very expensive and lacking a convincing method of assessing

their cost/benefit. As there is growing interest in Australia in the potential for re-stocking penaeid fisheries, it would clearly be advantageous to have some means of differentiating between introduced and wild prawns in order to monitor the effectiveness of reseeded and to fine-tune a release strategies.

The tags would ideally be: small enough to mark early life history stages; detectable at all subsequent life history stages; unique to the local population; able to identify individuals or cohorts; inexpensive and quick to apply and detect; either transmissible or non-transmissible to subsequent generations; and harmless to both the prawn and consumer (Rothlisberg and Preston, 1992). To this end, CSIRO conducted a FRDC funded pilot study to investigate the potential of two novel types of biological tags: reporter genes and chemical element markers (FRDC 92/07). The characteristics of these tags and the findings of the pilot study were as follows:

Reporter genes

New developments in genetic transformation in insects (*Drosophila*) offer the potential to create a novel type of benign genetic tag in the form of 'reporter genes'. The presence of these genes can be rapidly detected using a simple chemical test. In the pilot study we successfully micro-injected a reporter gene (phsp-CAT under the control of the *Drosophila melanogaster* hsp70 promoter) into prawn embryos and detected transient expression of the introduced enzyme within embryos. This was an encouraging preliminary step but further research was needed to progress beyond transient expression. Transient expression occurs when foreign DNA is transferred to the nucleus and transcribed outside the chromosome. The introduced sequences are amplified approximately tenfold in six to ten hours but usually are subsequently degraded during gastrulation (Stuart *et al.* 1988). Sometimes genes may persist in the animal by becoming incorporated into the host genome and thus become heritable by offspring, this process is termed stable integration.

One method that has been used very successfully to greatly enhance the efficiency of achieving stable integration in *Drosophila* is the use of transposable elements.

Transposable elements are discrete units of the host's own genetic material capable of moving from a reporter gene system to the host's chromosome at high frequencies (Spradling and Ruben, 1982). Hence, a foreign gene may be incorporated into the host genome with vastly improved efficiency over regular plasmids (bacterial DNA usually used to transport the reporter gene into the host). In order to capitalise on the results of our pilot study, the focus of the current study was to continue to develop an enabling gene transfer system for prawns including the potential of using of exogenous or endogenous transposable elements.

The detection and isolation of endogenous transposable elements could significantly enhance the development of enabling technology to achieve genetic transformation in prawns. Apart from providing precise, benign, biological tags, a genetic transformation system for penaeid prawns would provide a powerful technique for examining the underlying genetic, and biochemical processes controlling their reproduction, growth and defence against disease.

Trace elements

Variation in trace element composition has been successfully used to discriminate between fin-fish from different areas of Shark Bay in Western Australia (Edmonds *et al.* 1989). In crustaceans substantial differences in the concentration of elements occur in different body segments (Whyte and Boutillier, 1991). For example the hepatopancreas is a site of copper accumulation. The results of our pilot study demonstrated pronounced differences in the trace element composition of juvenile prawns collected from two widely separated drainage basins in the Gulf of Carpentaria, (FRDC 92/07.). The purpose of the current study was to determine whether the trace elements accumulated by juvenile prawns remain distinguishable in offshore adult populations and whether the trace element composition of prawns can be manipulated to provide a biological tag. The provision of a tag would require an element, or suite of elements, absent from the location of interest, that could be introduced via the diet of hatchery reared animals. These elements would need to be accumulated in prawn tissues, such as the nerves, and later be detectable in juvenile or adult prawns.

Objectives

- Determine the potential of gene transfer as a method of tagging penaeid prawns
- Determine whether transposable elements exist in the prawn genome
- Determine whether trace elements accumulated by juvenile prawns remain distinguishable in offshore adult populations
- Determine the uptake and residence time of selected trace elements in prawn tissues.
- Determine which body tissues of prawns best conserve the selected trace elements

Methods

Gene transfer

Source of prawn embryos

Mature *Penaeus japonicus* were obtained from the wild (offshore from Mackay) or from a commercial prawn farm (Moreton Bay Prawn Farm, Cleveland). The stock was maintained in 10 t seawater tanks maintained at 27°C and 35.8‰ salinity, under a reverse light cycle to ensure daytime spawning. Lights were switched on at 18.00 hrs and off at 06.00 hrs. The broodstock were fed frozen prawns and squid. Gravid females were induced to spawn by eye ablations. Spawning occurred in individual 50 l spawning tanks maintained at 29°C and 35.8‰ salinity. Embryos were collected by pipette approximately 15 minutes after spawning at the one-cell stage.

DNA manipulations:

The plasmids used were:

- p_{hsp}CAT: The bacterial chloramphenicol acetyl-transferase gene under the control of the *Drosophila melanogaster* hsp 70 heat shock promoter
- p_{hsp}GUS: The bacterial beta-glucuronidase gene under the control of the *D. pseudoobscura* hsp 82 heat shock promoter
- p_{hsp}GFP: The fluorescent green protein gene (GFP) from the jellyfish *Aequorea victoria* gene under the control of the *D. pseudoobscura* hsp 82 heat shock promoter
- pActCAT A: The bacterial chloramphenicol acetyl-transferase gene under the control of the *D. melanogaster* Actin 5C promoter.

- pActCAT B: The same as pActCAT A but with the gene in the reverse order. The gene is not functional in this orientation and this plasmid was used as a negative control.
- pActGUS: The bacterial beta-glucuronidase gene under the control of the *D. melanogaster* Actin 5C promoter
- pHFL1.supF: This plasmid contains a gene conferring ampicillin resistance and a modified hobo transposable element (HFL1), and a mutant tRNA gene (supF, Atkinson and O'Brochta, unpublished)

The DNA was stored as an ethanol precipitate (50ug-ampicillin^R) and contained a 1.8 kilobase *Bam* H1 fragment containing the reporter gene coding region subcloned into the *Bam* H1 site of pCaSperAct. Plasmids were precipitated with 70% ethanol and resuspended in sterile filtered sea water. Plasmid DNA was purified by centrifugation over two successive CsCl gradients. After purification and precipitation, the DNA was resuspended in TE buffer (Tris 10 mM, EDTA 1mM, pH 7.9) to a final concentration of 2 µg/µl

Transformations:

Transformations were performed with *Escherichia coli* strain DHS2 according to the protocol detailed in Manniatis *et. al.* (1982). The transformed bacteria were plated on SOB medium containing 20mM MgSO₄ and 50 µg /ml each of Kanamycin and Ampicillin (Boehringer Mannheim, Germany) and incubated overnight at 37 °C.

Large scale plasmid preparations:

A single colony was picked from the transformation plates and incubated overnight in 500 mls of L Broth with 50 µg /ml each of Kanamycin and Ampicillin. The cells were pelleted at 6K for 10 minutes and resuspended in 3 mls of Solution A (10mM EDTA, 25mM Tris-HCl pH 8.0), 6 mls of Solution B (0.2M NaOH and 1% SDS) was added, shaken to mix and stored on ice for 5 minutes, 4.5 mls of Solution C (3M sodium acetate pH 8.0 with glacial acetic acid) was added and the cells inverted to mix and stored on ice for 10 minutes. The cells were pelleted at 10K for 10 minutes and the supernatant retained.

The DNA was isolated from the supernatant by adding 2.5 times the volume of cold ethanol and placing at -20°C for 30 minutes. The DNA was pelleted at 10K for 10 minutes and the supernatant removed. The pellet was resuspended in 5mls TE buffer Ph 7.5 and transferred to Beckman centrifuge tubes (Rec #344075). 5.35g of caesium chloride (ICN Biomedicals) and 0.5 mls ethidium bromide (10mg/ml) was added and spun at 45K for 16 hours at 20°C .

The DNA bands were removed with a syringe (gauge 21). An equal volume of caesium chloride-saturated isopropanol was added, shaken and removed until the bottom layer was totally clear. The DNA was purified with ethanol as described previously and resuspended in sterile distilled water. The amount of DNA was measured spectrophotometrically at 260nm.

Microinjection

Two means of immobilising embryos for microinjections were used. Initially, embryos were drawn into silastic tubing with an internal diameter of 0.5 mm and their movements were controlled by regulating water flow through the tubing. A small hole was cut in the tubing into which a glass injection needle could be inserted. This method was effective but time consuming and the embryos would develop beyond the 1 or 2 cell stage before many embryos could be injected. Consequently, we devised a more efficient method of securing the embryos in which embryos were simply pipetted onto a taut $250\ \mu\text{m}$ nylon mesh (Swiss Screens, Brisbane) that was immersed in sterile seawater. Since the embryos have a diameter of $300\ \mu\text{m}$, they were held securely on the grid of the mesh. Glass injection needles were prepared using a Flaming Brown Needle Puller (Sutter Instruments), and the needle tips were sharpened using a needle beveller (Narashige Instruments). DNA flow through the needle was regulated by a Pneumatic Pico-Pump (World Precision Instruments, USA) that was connected to a pressurised helium source. A 100 ms pulse at 18 psi was applied to each embryo. Injected embryos were washed into sterile seawater. Control embryos were injected with injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8) containing no DNA.

Electroporation:

Two methods of electroporation were used. The first series of experiments on DNA delivery by electroporation were done using an electroporation apparatus designed and built by CSIRO Division of Plant Industry, Canberra. This apparatus delivered 1 pulse of 0-500V with settings of pulse lengths from 100 μ s to 10ms. The electroporator delivered a square pulse over the pulse length. Embryos were electroporated in a 1ml spectrophotometer cuvette into which aluminium electrodes with a 0.7cm gap were inserted. Prior to electroporation the embryos were rinsed in sterile seawater, and up to 100 were removed in 400 μ l and added to a cuvette. Following electroporation, the embryos were allowed to recover for 10 minutes on ice and then placed in 100 mls of filtered sea water for overnight incubation at 25°C. After 20 hours, hatch rates were calculated as a percentage of the control.

To determine embryo survival rates versus potential DNA entry, we investigated the effects of variation in electric field strength (V/cm) and pulse length (ms).

Electroporation trials were done with two reporter gene systems, CAT and GUS. 10 μ g of the required DNA vector system was placed into a cuvette containing approximately 100 embryos. The embryos were then subjected to a 67 V/cm pulse for 1 ms using one pulse. Negative controls included embryos without DNA for one pulse, and embryos without DNA and no pulses. A typical CAT expression assay consisted of two negative controls (no DNA and pActCAT B), pHspCAT A with one pulse and a positive control with the enzyme but no DNA.

The second series of experiments on DNA delivery by electroporation was performed using a slot cuvette technique previously described (Leopold et al., 1996). A portion of the embryos were collected from spawning tanks containing 0.1 mM 3-amino-1,2,4 triazole (ATA). ATA is a peroxidase inhibitor, and has been demonstrated to reduce hardening of the hatching envelope in other penaeid prawn embryos (Lynn et al., 1993). Approximately 100 one- to four-celled embryos were placed between two aluminium plates that were fixed to a microscope slide 2 mm apart. Most of the seawater surrounding the embryos was removed and 40 μ g/ml plasmid DNA in sterile seawater was added. Control embryos were subjected to electroporation in seawater containing no DNA. Electrical charge was applied via alligator clamps attached to the leads from an Electro Cell Manipulator (BTX 600). Following the electrical pulse, embryos were

washed into sterile seawater containing 0.2 µg/ml chloramphenicol and allowed to develop. Initial experiments were conducted to determine the effects of different voltage, resistance and capacitance on the survival of embryos. These trials demonstrated that 1 cell stage embryos were too sensitive for electroporation, with less than 10% survival. Hence, all subsequent experiments were performed on embryos at the 2 to 4 cell stage. Optimisation for survival resulted in a protocol of an electrical pulse of 40 V, a resistance of 24 ohms, and a capacitance of 300 µF. These conditions resulted in survival rates of approximately 75% for untreated embryos and 35% for ATA-treated embryos.

Bombardment

Gold particles with a diameter of 1.6 µm (Biorad, California) were prepared for DNA coating by sonication and repetitive washing. Particles were washed three times in 100% ethanol and sonicated for 1 minute. The particles were precipitated by brief centrifugation, and then washed twice in sterile distilled water. The particles were resuspended in 50% glycerol at a concentration of 100 mg/ml and stored in aliquots of 25µl at -20°C. Plasmid DNA was bound to the gold particles according to a method previously described (Sanford, 1988), and 2 µg DNA coated to 25 µg gold particles were used for each bombardment.

The bombardment apparatus was a conventional helium gas driven particle bombardment gun with propulsion at a pressure of 90 kPa into a sample chamber was connected to a vacuum pump. DNA-coated gold particles were placed on a 13µm Swinney filter (Millipore, USA), and connected to a filter holding attachment. Approximately 400 one-celled embryos were placed on a filter paper and were bombarded under a vacuum of 500 mm Hg, at distances ranging from 45 to 95 mm from the particle delivery source (gun). After bombardment, the embryos were given 3 successive washes to remove any gold particles that had not penetrated the embryos, and were then placed in sterile seawater containing 0.2 mg/ml chloramphenicol. Embryos subjected to bombardment of particles with no bound DNA served as negative controls.

DNA expression assays

Beta-Glucuronidase staining:

Micro-injected or electroporated embryos were collected at the mid-blastula or nauplii stage of development and ground in a glass tissue macerator in 10mM sodium phosphate pH 8.0. The tissue was transferred to 100 μ l staining solution (50 μ l 1M sodium phosphate, 20 μ l 100mM potassium ferricyanide, 20 μ l 100mM potassium ferrocyanide, 50 μ l 20mg/ml 5-Br-4-Cl-3-Indolyl-B-D-Glucuronidase (X-Glu) and 860 μ l 5% Ficoll) in wells of a microtitre plate. The wells were sealed and placed at 37 °C overnight. The assay is positive when a blue colour has developed.

CAT enzyme assay:

The assay was carried out by two separate methods in accordance to the supplier's protocol (Promega) with two modifications. The first modification was to collect the embryos just prior to hatching and grinding them with a glass macerator. DNA was extracted in Tris buffer pH 8.0. 50 μ l of the extract was reacted with 68 μ l 0.25M Tris-HCl Ph 8.0, 2 μ l ¹⁴C-Chloramphenicol (at 0.025 mCi/ml) and 5 mg/ml n-butyryl Coenzyme A and allowed to incubate overnight. Reactivity was assessed using the LSC assay system.

The second modification was to collect developed embryos and place them in microfuge tubes at -80°C for 15 minutes. Embryos were then ground with a glass tissue macerator in 100 μ l 0.25M Tris-HCl pH 7.7 and placed at -80°C for 10 minutes. The cell extracts were exposed to 65 °C for 5 minutes and spun in a bench top centrifuge (Tomy HF120, Quantum Scientific) for 10 minutes at 4°C. The supernatant was retained and 50 μ l was allowed to react according to the preceding protocol for one hour at 37°C. 1ml of ethyl acetate was then added and spun to achieve phase separation. The upper phase was retained and allowed to dry overnight. The pellet was dissolved in 10 μ l ethyl acetate and was spotted onto a silica thin layer chromatography (TLC) plate (Alltech, Sydney). The plate was run for 15 minutes in 1:20 methanol:chloroform. Once dry, the plate was sprayed with 0.4g PPO dissolved in 100mls 1-methyl- naphthalene and exposed to an X-ray film (Fuji) overnight and developed.

Plasmid delivery detection

Plasmids were recovered from developing embryos 3 or 24 hours post-DNA delivery. For each of the electroporation and microinjection experiments, half of the developed

embryos were treated with 0.01 mg/ml DNase I (Promega) for 30 min to remove any plasmid DNA that might be adhering to the exterior of the embryos. The DNase was inactivated by a 2 h treatment with 0.1 µg/ml Proteinase K at 37°C, followed by two successive washes in sterile sea water. Embryos and nauplii were ground in 100µl homogenization buffer (0.5% SDS, 0.08M NaCl, 0.16M sucrose, 0.06M EDTA and 0.12M Tris-HCl, pH9.0) and incubated at 65°C for 30 minutes. Potassium acetate was added to a final concentration of 1M and the samples were placed on ice for 30 minutes to precipitate the proteins. The samples were centrifuged and the supernatant retained. The DNA was precipitated in ethanol, and the pellet resuspended in 10ul TE buffer. The rescued DNA was electroporated into DH10B cells using an Electro Cell Manipulator (BTX, USA) according to the manufacturer's protocol. The transformed bacterial cells were incubated in SOC media (Sambrook et al., 1993) for 1 h at 37°C and were spread onto agar plates containing ampicillin. The number of ampicillin-resistant colonies growing on the plates the following day were counted and recorded.

Screening of genomic DNA for transposable elements

Details of the methods used for polymerase chain reaction screening of genomic DNA for *hobo*-like elements are given in the provisional Australian patent on transgenesis in crustaceans (PN4593).

Mitochondrial and microsatellite DNA markers

Mitochondrial DNA variation in *Penaeus japonicus*

As noted in the progress report (June 1994) this preliminary study was done by Dr S. Lavery (Queensland Agricultural Biotechnology Centre). In order to determine the levels of genetic variation that could be detected among *Penaeus japonicus* individuals, mitochondrial DNA (mtDNA) was examined in a number of wild-caught and pond-reared individuals. The study examined variation in two mtDNA segments using direct DNA sequencing of segments amplified using PCR (polymerase chain reaction).

Total purified DNA was extracted from 62 samples, comprised of 50 wild-caught individuals and 12 samples of eggs, nauplii or tissue from artificially (pond) reared

individuals. The quality and concentration of each extraction was checked on agarose gels and by spectrophotometry. PCR primers were tested for their ability to amplify segments of *P. japonicus* mtDNA. The primer pairs chosen amplify segments of the gene encoding the large 16s ribosomal RNA subunit (16s). The 16s fragment was sequenced in 12 individuals (7 wild, 4 pond and one from China), giving a 500bp of sequence from each.

Microsatellite DNA markers in *Penaeus esculentus*

As noted in the progress report (June 1994) this preliminary study was done by Dr S. Moore (CSIRO Tropical Agriculture). At the time of this study the isolation of DNA sequences on structural regions termed microsatellites was relatively new technique for determining genetic variation between individuals, families or species. The methods developed by Dr S. Moore for the isolation of microsatellites from *P. esculentus* are unpublished and the property of CSIRO (for further information contact Dr S. Moore).

Trace elements

Following analysis of data obtained in the FRDC pilot study on the development of biological tags (92/037) we decided not to pursue the first objective to:

- Determine whether trace elements accumulated by juvenile prawns remain distinguishable in offshore adult populations

We abandoned this objective because the results of the pilot study showed that trace element composition was highly variable within and between prawn tissue types and between seasons, locations, diets and sediments. Given these results it would have been premature to attempt (as planned) to determine whether trace elements accumulated by juvenile prawns remain distinguishable in adult populations. Instead we focused our efforts on the other two objectives of the project, which were to determine:

- the uptake and residence time of selected trace elements in prawn tissues
- which body tissues of prawns best conserve the selected trace elements

The uptake and residence time of selected trace elements in prawn tissues
Based on the results of the pilot study (FRDC 92/037) we selected selenium from among the trace elements that accumulate in the tail muscle of juvenile prawns. Initially we examined the uptake, retention and toxicity of sodium selenate ($\text{Na}_2\text{O}_4\text{Se}$) and selenite ($\text{Na}_2\text{O}_3\text{Se}$) in juvenile *Penaeus japonicus* (0.02 - 0.7 g total wet weight) in the laboratory. Prawns (initial wet weight 0.02 g) were fed artemia mixed with either low (2 ppm) or high (5 ppm) concentrations of sodium selenate or sodium selenite for a period of 6 weeks. The prawns were then fed artemia alone (no added selenium) for a further 6 weeks. The prawns were reared in 90 l nylon bins with flow-through seawater maintained within a diurnal temperature range of 23°C to 25 °C at ambient salinity (33‰). The experimental design consisted of 12 tanks. There were three replicate tanks, each containing 50 prawns, for each of the two treatments; high (5 ppm) and low (2 ppm) selenium concentrations. The prawns in the three control tanks were fed artemia alone (no added selenium). Samples of prawns were collected every two weeks and immediately frozen. The concentration of selenium retained by the prawns was determined by inductively coupled plasma mass spectrometry (ICPMS).

Which body tissues of prawns best conserve the selected trace elements

In order to determine which body tissues best conserve selected trace elements we used a diet enriched with rare stable isotope of selenium (^{74}Se) to monitor uptake and retention. In the experimental design there were 8 tanks, each containing 50 prawns. There were four replicate treatments fed the enriched selenium diet and 4 control tanks fed the same diet but with no added selenium. Samples of 6 individual prawns taken at random from within the treatments and controls were collected every 2 weeks. The concentration of the (^{74}Se) retained by the prawns was determined by inductively coupled plasma mass spectrometry (ICPMS).

Tagging experiments using rare isotopes of common elements ^{15}N and ^{13}C

Phytoplankton ^{15}N and ^{13}C enrichment

Monocultures of the marine diatom *C. muelleri* were progressively cultured from starter cultures of 200 ml through 4, 20, 2000 and finally to 5000 l. Starter cultures were grown up

to the 20 l stage (concentration 1×10^6 cells ml^{-1}) in f/2 nutrient media (Guillard, 1983) at ambient temperature (24 to 25°C) and salinity (32 to 35‰) with constant illumination (fluorescent light). From the 20 l stage, cultures were transferred initially to a 2000 l indoor tank and finally to a 5,000 l tank. The tanks were illuminated with natural light through a transparent (alcyonite) roof. The nutrient medium added to the 2000 l tank was: KNO_3 (75 mg l^{-1}), $\text{Na}_2 \text{HPO}_4$ (5 mg l^{-1}) and $\text{Na}_2 \text{SiO}_3$ (2.5 mg l^{-1}). The nutrient medium added to the 5,000 l tank was: KNO_3 (20 mg l^{-1}), $\text{Na}_2 \text{HPO}_4$ (2.5 mg l^{-1}) and $\text{Na}_2 \text{SiO}_3$ (2 mg l^{-1}). In the nutrient medium for the 5,000 l tank, 5% of the KNO_3 added had enriched ^{15}N (99.4 % pure, Cambridge Isotope Laboratories, Andover, MA, USA) and an approximately equivalent concentration of ^{13}C was added in the form of $\text{Na C}^{13} \text{O}_3$ (99.4 % pure, Cambridge Isotope Laboratories, Andover, MA, USA). The cultures were grown to a density of $>0.6 \times 10^6$ cells ml^{-1} within a diurnal temperature range of 23 to 25°C at ambient salinity (33‰).

Artemia ^{15}N and ^{13}C enrichment.

A 5,000 l culture of ^{15}N enriched *C. muelleri* was prepared as described above. At the same time *Artemia* were hatched in a 2,000 l tank of seawater maintained at a temperature of 28 °C and a salinity of 33‰. Following hatching, the *Artemia* nauplii were separated from their cysts by light attraction. Approximately 20 hours after hatching *Artemia* were fed the ^{15}N enriched diatoms at an initial concentration of 10 *Artemia* ml^{-1} and 0.6×10^6 diatoms ml^{-1} . *Artemia* were harvested after 24 hours which ensured sufficient time for total grazing of the algal cells and evacuation of undigested remains. After harvesting, *Artemia* were rinsed thoroughly with filtered (1 μm) freshwater to remove salt and any residual algal cells. The ^{15}N and ^{13}C enriched *Artemia* paste was then freeze dried.

Prawn ^{15}N and ^{13}C enrichment and depletion.

In the experimental design there were 7 x 90l tanks. Each tank was initially stocked with 100 post-larvae *P. esculentus* prawns. The experiment ran for a total of 12 weeks. From

weeks 1 to 6 during the uptake, prawns in 4 of the tanks were fed the labelled carbon-nitrogen diet, and those in the other 3 tanks were fed a control fed the same amount of unlabelled *Artemia*. At 10:00 each morning the tanks were fed 0.2g of frozen labelled *Artemia*, and the water flow to the tanks turned off. At 14:00 the animals were fed a non-labelled commercial diet (PL+500 Frippak), and the water turned on again. This feeding method continued until week 6. After week 6, tanks prawns in two of the tanks were taken off the labelled diet and fed unlabelled artemia plus the commercial diet. Two tanks remained on the labelled *Artemia* plus the commercial diet.

From weeks 1 to 6, samples of prawns from each tank were taken every week. From weeks 6 to 12 samples were taken every 2 weeks. The sampled prawns were placed in a container of fresh seawater and held overnight to permit the clearing of their guts. They were rinsed in freshwater and frozen the following morning. All samples were then oven dried at 60°C overnight and then ground with mortar and pestle. Ground samples were weighed into tin capsules and passed through a Europa Scientific Roboprep for oxidation. The resultant nitrogen from each sample was analysed for $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$ ratios and percent nitrogen with a continuous flow isotope ratio mass spectrometer (Europa Tracermass, Crewe, U.K.).

Results

Gene transfer

Delivery of DNA to prawn embryos

Microinjections

Microinjections provided the most effective and consistent means of delivering DNA into the embryos. Using either the tube or mesh method of immobilising the embryos, approximately 500 times more plasmids were recovered following microinjection compared to that recovered after electroporation (Table 1). With the mesh immobilisation method, considerably more embryos could be injected in the limited time before the embryos developed beyond the 4 cell stage. Treatment of the microinjected embryos with

DNase did not affect plasmid recoveries following microinjection, which suggests that, unlike the other techniques, most DNA was internalised by the injection method. The number of plasmids persisting in developing embryos over a 24 h period was also assessed following microinjection. Recovery of plasmids after 24 h development dropped by approximately 33% from those levels observed after 3 h development, indicating that while some DNA may be degraded or lost from the embryo over the 24 h period, most of the plasmid still persisted in the embryonic cells (Table 2).

Table 1

Recovery of plasmids 3 h post-microinjection of *Penaeus japonicus* embryos. The values represent the mean +/- standard deviation of plasmids recovered from embryos injected with different methods (tubing or mesh) with or without DNase treatment after microinjection.

Treatment	DNase treatment	# embryos injected	plasmids/embryo ²	cell stages injected
no DNA	-	120 ¹	0.03 (0.02)	1-4
embryos in tubing	+	25	430 (113)	2-4
embryos on mesh	-	146 ¹	209 (55)	1-4
embryos on mesh	+	123 ¹	225 (25)	1-4

¹ values represent approximately 50% of embryos injected, as the remaining 50% were permitted to develop for 24 h.

² values are the means and (standard deviations) of three independent experiments

Table 2

Recovery of plasmids from microinjected embryos 3 and 24 h post-microinjection. The values represent the mean +/- standard deviation.

	Plasmids/embryo ¹		% decrease
	3 h post injection	24 h post injection	
no DNA	0.03 (0.02)	0.04 (0.02)	-
expt 1	243 (32)	160 (25)	34.2
expt 2	197 (34)	122 (18)	38.1
expt 3	235 (26)	176 (18)	25.1

¹ values represent the mean and (standard deviation) from 3 replicate plates

Electroporations

Although significant numbers of plasmids could be recovered from non-DNase-treated embryos, treatment of the embryos with DNase prior to plasmid rescue indicated that greater than 99% of the DNA was susceptible to DNase digestion (Table 3).

These results suggest that most of the electroporated DNA was bound to the exterior of the embryo. In contrast, treatment with DNase did not affect plasmid recoveries from microinjected embryos (see above), which indicates that the DNase treatment was not digesting DNA that had apparently entered the cells. Despite these sizeable losses of DNA on the exterior of the embryos, a significant number of plasmids above background levels could be recovered from electroporated embryos.

Table 3

Plasmid recoveries from 2 to 4 cell stage *Penaeus japonicus* embryos subjected to electroporation, treated with and without ATA and DNase. The values represent the mean +/- standard deviation for 3 separate experiments. Letters denote significant differences (0.01 level) between values

Embryo treatment	DNase treatment	plasmids/embryo	Sig.
no DNA	-	0.05 (0.02)	a
no ATA	-	123 (64)	b
no ATA	+	0.5 (0.2)	a
ATA	-	110 (52)	b
ATA	+	0.9 (0.3)	c

In an effort to improve DNA penetration into the embryos, spawned embryos were exposed to ATA to reduce hardening of the hatching envelope. Recovery of plasmids from ATA-treated embryos however, showed no significant difference from untreated embryos. No plasmids were recovered from electroporated embryos or hatched nauplii that had developed for 24 h.

Bombardment

Survival rates of embryos subjected to particle bombardment were inversely proportional to the distance from the delivery source (Figure 1). No survivors were observed for embryos placed at the shortest distance from the gun (45 mm), whereas at the farthest distance tested (90 mm), survivorship was 85%.

As maximum survival was indicative that no gold particles were penetrating the embryos, and no survival indicated severe damage due to penetration by too many particles, we selected an intermediate distance of 65 mm for the DNA delivery experiments. Plasmid recovery was only attempted for embryos that underwent further cell divisions following bombardment. The number of plasmids recovered per embryo rapidly diminished with successive washes of the embryos prior to plasmid rescue (Table 4), which indicates that most of the gold particles attached to the surface of the hatching membrane. After three successive washes the number of plasmids detected was not significantly different from embryos bombarded with particles with no bound DNA (Table 4).

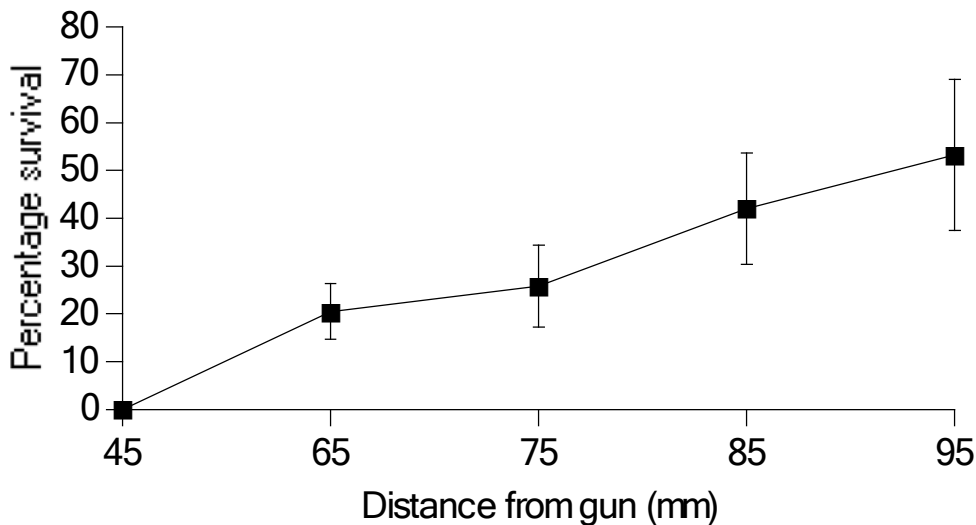


Figure 1. The effects of gun distance on the survival of 1 to 2 cell stage embryos of *Penaeus japonicus*.

Table 4. Plasmid recoveries from embryos subjected to biolistics, treated with and without ATA and DNase. The values represent the mean +/- standard deviation for 3 separate experiments. Letters denote significant differences (0.01 level) between values

Embryo treatment	plasmids/embryo	Sig.
no wash	320 (32)	A
first wash	20 (8)	B
second wash	12 (8)	B
third wash	0.3 (0.02)	C
no DNA	0.1 (0.02)	C

Expression of reporter genes in prawn embryos

Using microinjection, the fluorescent green protein (GFP) gene from the jellyfish *Aequora victoria*, the bacterial chloramphenicol acetyl-transferase (CAT) gene and the bacterial beta-glucuronidase gene were all found to be expressed in detectable levels in developing embryos or nauplii of *P. japonicus* (Table 5). Expression was affected either via the hsp70 promoter from *Drosophila melanogaster* or the hsp82 promoter from *D. pseudoobscura* which, in related studies, has been found to be constitutively expressed in insects other than *D. pseudoobscura*.

Table 5. Reporter gene expression in embryos or nauplii of *Penaeus japonicus* following microinjection or electroporation at the one or two cell stage

<i>Construct</i>	<i>Delivery method</i>	<i>Response</i>
phspCAT	Microinjection	expression in embryos and nauplii
phspCAT	Electroporation	expression in embryos
phspGUS	Microinjection	expression in embryos
phspGUS	Electroporation	no expression
phspGFP	Microinjection	expression in embryos and nauplii
phspGFP	Bombardment	expression in embryos and nauplii
pactCAT A	Microinjection	no expression
pactCAT B	Microinjection	no expression
PActGUS	Microinjection	no expression
PactGUS	Microinjection	no expression

Using electroporation, CAT gene expression affected via hsp70 promoter was detected in embryos but no GUS (hsp82) expression was detected. Using bombardment, GFP (hsp82) gene expression was detected in developing embryos and nauplii of *P. japonicus*. No detectable levels of CAT or GUS activity were found when the genes were under the control of the *D. melanogaster* Actin 5C promoter.

Transposable elements in the prawn genome

Polymerase chain reaction screening of genomic DNA for *hobo*-like elements gave positive results for *Penaeus esculentus*. The results showed that *P. esculentus* contains sequences which hybridise to the *hobo* element. Details of the conditions used for DNA amplification are given in the provisional Australian patent on transgenesis in crustaceans (PN4593).

Discussion

The first step in generating transgenic organisms is the introduction of foreign DNA into a suitable life-stage of the organism. Microinjection of DNA into early stage embryos has already resulted in the development of a broad range of transgenic organisms, including insects (Rubin and Spralding, 1982) sea urchins (McMahon et al., 1985), fish (Chen and Powers, 1990) and mice (Palmiter and Brinster, 1986). Our study has demonstrated that microinjections are also an effective means of introducing DNA into prawn embryos. Although microinjections are more time-consuming than either electroporation or particle bombardment, the efficiency of this technique relative to the others confirms its merits. Unlike electroporation and particle bombardment, microinjection ensures that the DNA enters the cells, as we demonstrated with DNase treatment of the prawn embryos following DNA introduction. Given the efficacy of this procedure, sufficient numbers of embryos could be injected to attempt transient expression assays, where various promoter and marker gene constructs could be tested in prawn embryos. In addition, this technique will permit efforts to develop a genetic transformation system for penaeid prawns.

Microinjection has been the most widely used technique to introduce DNA into animal embryos but electroporation has recently been used to introduce DNA into embryos of a few insect species (Kamdar et al., 1992; Leopold et al., 1996), fish (Muller et al., 1993; Tsai and Tseng, 1994) and abalone (Powers et al., 1995; Counihan et al., 1997). From these few cases, it is evident that electroporation conditions must be varied considerably in order for the DNA to penetrate the different egg shells and extra embryonic membranes of each species. Partial removal of the hatching envelope in *P. japonicus* using ATA was not sufficient to permit significantly more penetration of the DNA into embryos under the conditions tested. Further studies varying pulse number, strength, and duration could result in considerably more DNA entering the embryos, and future studies will be directed to this goal.

Although particle bombardment proved unsuccessful in transfecting *P. japonicus* embryos, the technique has been successfully applied to embryos of another marine crustacean (Gendreau *et al.*, 1995) and in fish (Zelenin *et al.*, 1991). In the former case, DNA was successfully introduced into 20 h old *Artemia* embryos, but no DNA could be introduced into younger dechorionated embryos. Presumably the other extra embryonic membranes of younger embryos were too tough to be penetrated by the particles under the conditions tested. *P. japonicus* might similarly have a limited period of development that can both withstand the bombardment and still permit particle entry into the developing embryos.

Plasmid rescues are a particularly sensitive means of measuring the amount of DNA that has entered a cell. Transient expression of reporter genes can also provide this information, but in a new species, there can be uncertainty as to whether the selected promoters or marker genes will work in a heterologous system. Having established an effective means of introducing DNA into *P. japonicus* embryos, we can now begin testing various heterologous promoters and marker genes in anticipation of developing a genetic transformation system in this species. Contingent upon the implementation of a genetic transformation technique, our preliminary results indicate that CAT, GUS and GFP could be used as markers in penaeid prawns.

Transposable elements such as *P*, *hobo* and *mariner* have been successfully used as transformation vectors in the fruit fly *Drosophila melanogaster*. The development of this technology has rapidly improved our knowledge of the basic biology of this insect. The success of transposable elements in achieving gene transfer in *D. melanogaster* suggests that this approach may form a basis for successful gene transfer technology in other invertebrates including penaeid prawns.

Ultimately, genetic transformation will provide a powerful technique for examining the underlying biochemical and genetic processes of reproduction and defence against disease. The most significant application of this technology to penaeid prawns could be in genetically engineering traits such as improved growth or resistance viral diseases.

Mitochondrial and microsatellite DNA markers

Mitochondrial DNA variation in *Penaeus japonicus*

Among the Australian samples of *P. japonicus* mtDNA, 3 sites (0.6%) proved polymorphic, identifying 4 different haplotypes, only two of which were found among the pond individuals (Table 6). The inferred phylogenetic relationships among the 4 haplotypes are shown in Fig. 2. One of the three polymorphic sites (site 239) could be detected using a restriction enzyme (*MaeIII*), and this was used to assess variation among all 62 samples at this site. One wild and three pond samples were found to possess that restriction site, which identified them as having haplotype C. The other polymorphic sites could also be screened in the entire sample by using specific primers which introduce a restriction site.

The one individual from China that was examined for 16s rRNA sequence proved to be extremely different from the Australian samples. Ten additional nucleotide site differences (2.0%) were observed in the 16s fragment.

Table 6. Mitochondrial 16s rRNA haplotypes of 12 *P. japonicus* individuals.
 * 1-7 were wild-caught, 59-62 pond-reared, and CH was from China. # Haplotype designation.

ID*	Haplo. #	S I T E											
		26	98	129	146	147	231	239	275	297	341	342	415
1	C	T	C	A	A	G	T	C	A	G	A	C	T
2	B		C					T		G			
3	D		T					T		A			
4	B		C					T		G			
5	A		T					T		G			
6	A		T					T		G			
7	A		T					T		G			
59	A		T					T		G			
60	C		C					C		G			
61	A		T					T		G			
62	A		T					T		G			
CH	E	C	T	C	G	A	C	T	G	G	G	T	C

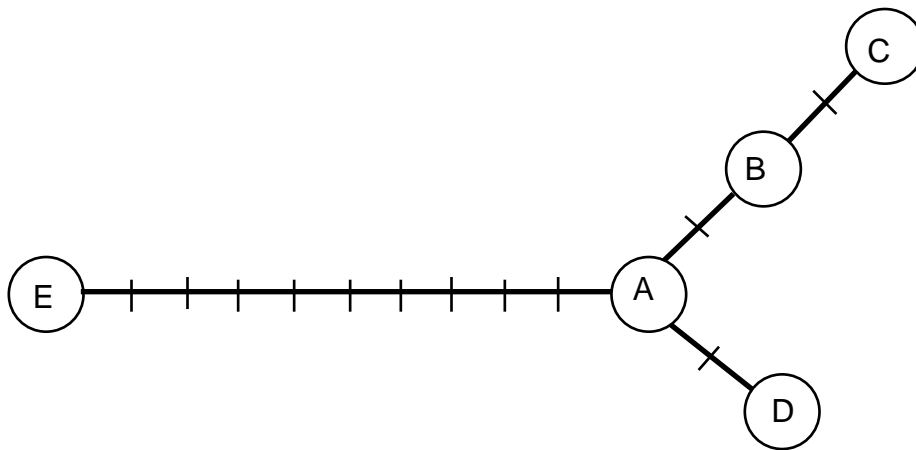


Figure 2. Phylogenetic relationships among *Penaeus japonicus* mtDNA haplotypes

Microsatellite DNA markers in *Penaeus esculentus*

The preliminary characterisation of microsatellite markers in *P. esculentus* has demonstrated the presence of species specific primer binding sites (Fig 3). The results also demonstrated that microsatellites in *P. esculentus* are locus specific polymorphic markers (Fig 4).

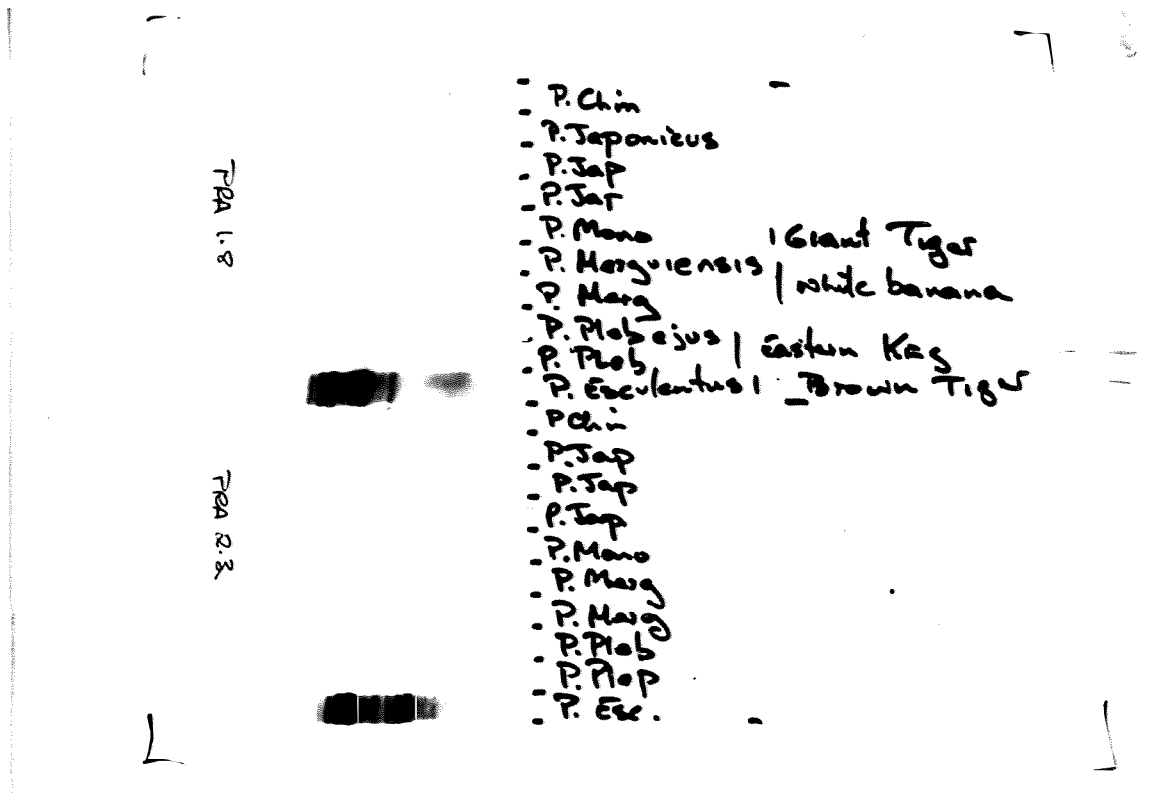


Figure 3. Specific primer binding sites in *Penaeus esculentus*

Disussion

Collaboration with the CSIRO Division of Tropical Agriculture and the Queensland Agriculture Biotechnology Centre has enabled us to assess the potential of using natural genetic markers (mitochondrial DNA and microsatellites) as biological tags for penaeid prawns. These preliminary results have shown that variation in mitochondrial DNA permits a higher level of discrimination between prawn stocks than allozyme techniques. The results obtained for *P. japonicus* mtDNA showed that a considerable level of genetic variation could be detected among individuals using this technique.

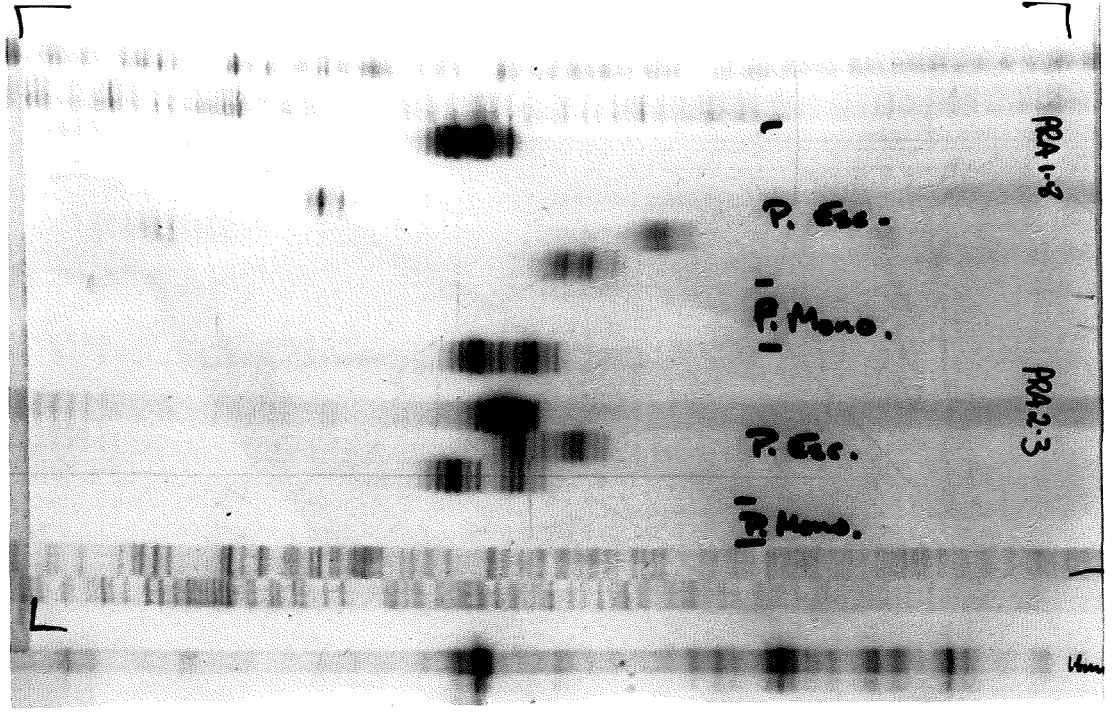


Figure 4. Alleles in individual *Penaeus esculentus* (seven alleles in four individual)

The haplotype diversity of approximately 70% compares with an allozyme heterozygosity of around 2% from other penaeid prawn species. Of considerable importance is the fact that the bulk of this DNA variation can be quickly and easily screened in large numbers of individuals using simple restriction enzyme techniques. Screening, isolation and amplification of rare mitochondrial variants could be used as means to tag prawns, particularly if used in conjunction with microsatellite markers.

Preliminary characterisation of microsatellite markers in *P. esculentus* demonstrated the presence of species specific, locus specific polymorphic markers. Microsatellite markers may provide a higher level of discrimination among prawn stocks than mitochondrial DNA and have considerable potential as natural tags in stock-enhancement programs.

The use of mitochondrial and/or microsatellite variants would require extensive screening of the natural population and, in most cases, amplification of the variant(s) through one or more captive generations.

In any large scale reseeded program care needs to be taken to ensure that the genetic diversity of the enhanced population is maintained. The choice of genetic markers to use in screening the natural population will depend on what is available for the species of interest. There are a number of possible options for genetic markers to use in both screening and/or tagging prawns for reseeded programs. Three different types of DNA markers have already been developed for penaeid prawns; mitochondrial (see above and Benzie *et al.* 1993), microsatellites (see above and Moore *et al.* in press) and Amplified Fragment Length Polymorphisms (Moore *et al.* in press). In *Penaeus japonicus* twelve microsatellite loci displayed between 4-24 alleles and heterozygosities between 50 and 90 percent in wild sourced animals. Amplified Fragment Length Polymorphisms (AFLPs) exhibit high levels of variation in *P. japonicus* with over 570 polymorphic loci being defined using 40 different combinations of primers. The high level of variation and relative ease of isolation and characterisation of AFLPs suggest that these may be markers of choice for screening and monitoring genetic diversity in prawn reseeded programs

The use of natural genetic variants as tags requires the selection of rare DNA variants from the natural population and a breeding program to produce the required numbers of individuals for reseeded. This approach raises concerns about reducing the genetic diversity of the natural population. One potential method of addressing this problem would be use rare mitochondrial DNA female siblings mated with different wild males. Because mtDNA is maternally inherited the progeny would all carry the rare mtDNA allele but could be highly diverse for nuclear DNA. Alternatively, a careful breeding program could produce large numbers of offspring homozygous for a rare nuclear gene (with a natural frequency of only q^2) while maintaining diversity at other loci. Screening and isolating rare alleles and then breeding sufficient individuals for reseeded is both costly and time consuming. An alternative approach could be to introduce a 'unique'

genetic tag in the form of a benign reporter gene. This approach would permit the use of the required number of parents and progeny to maximise the maintenance of natural genetic diversity. However, although techniques for the introduction of reporter genes are now well advanced in penaeid prawns (Preston *et al*^bin press) stable transformation is yet to be achieved. Furthermore, current concerns about any release of any genetically modified organism to the marine environment means that the use of such an approach remains a remote, but intriguing prospect (Rothlisberg *et al.* in press).

Trace elements

The uptake and residence time of selected trace elements in prawn tissues

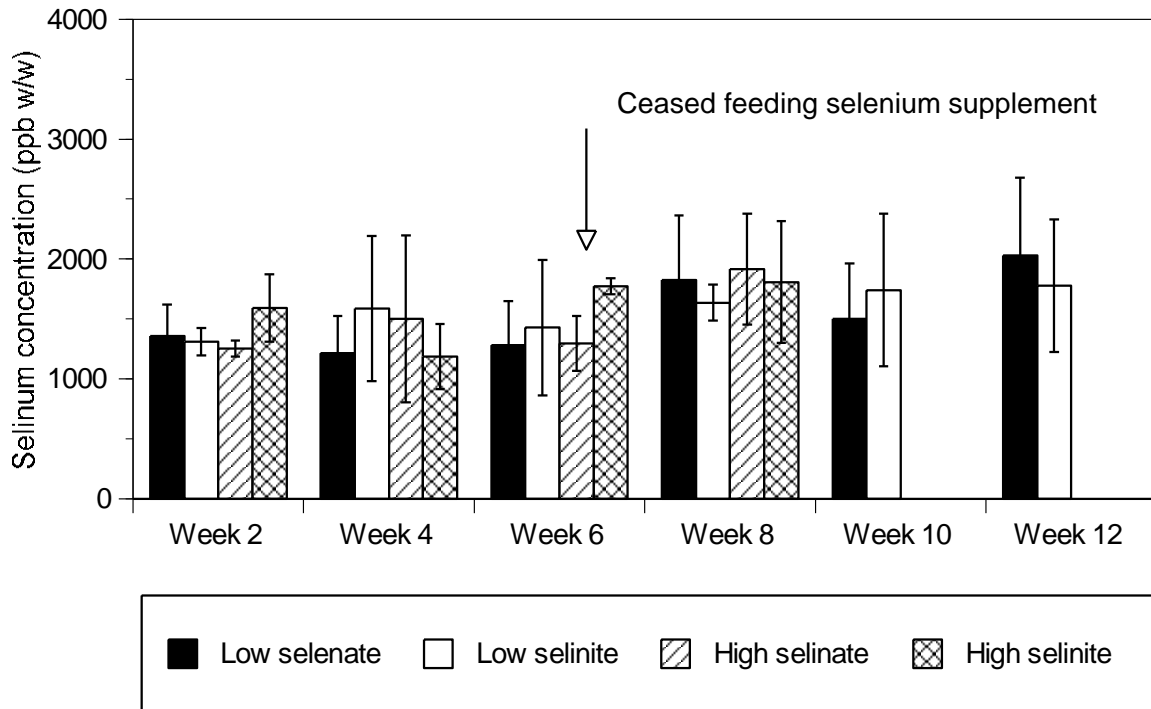


Figure 5. Variation in concentration of selenium in the abdominal muscle of juvenile *Penaeus japonicus* fed low (2 ppm) and high (5 ppm) concentrations of sodium selenate or selenite for a period of 6 weeks and then maintained on a control diet for a further 6 weeks.

The results showed that juvenile prawns maintain a relatively constant level (mean values: 1.2 - 2.2 ppm) of selenium in their abdominal muscle (Fig. 5).

Analysis of variance demonstrated that there was no significant difference in the muscle selenium content of prawns fed low (2 ppm) or high (5 ppm) concentrations of sodium selenate or sodium selenite for a period of 6 weeks (Fig 5).

All the prawns that were fed high concentrations of sodium selenate or sodium selenite died after 8 weeks. After supplementary additions of selenium ceased the level of selenium in juvenile prawn muscle remained constant until the end of the experiment (Fig. 5).

Variation in trace elements assimilation in different body tissues

The results of the pilot study of wild-caught juvenile *P. merguensis* showed that higher concentrations of most trace elements were found in the mid-gut gland (hepatopancreas) of than in other tissue types (FRDC 92/07). Conversely, the abdominal muscle appeared to be the most conservative tissue type in retaining trace elements. The pilot study also showed that arsenic, chromium and selenium all accumulate in the abdominal muscle of juvenile prawns.

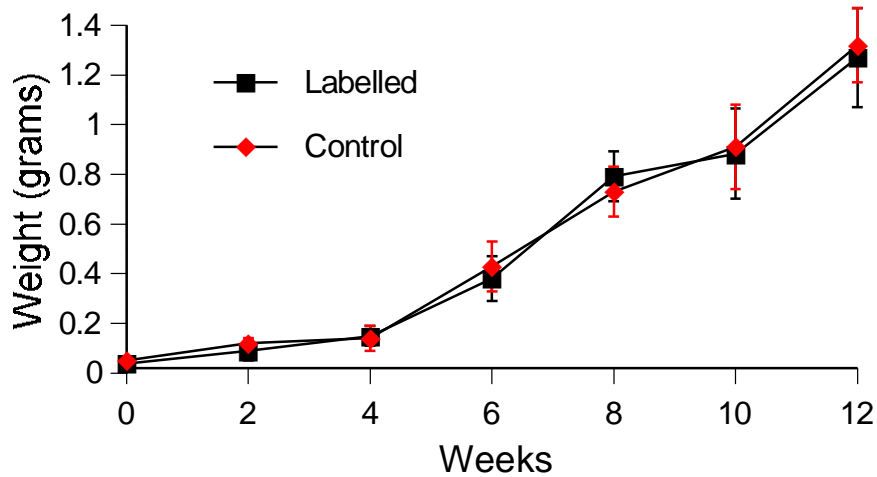


Figure 6. Comparisons of the growth of juvenile *Penaeus japonicus* fed enriched ^{74}Se (labelled) and those fed a control diet without enriched ^{74}Se . The feeding of enriched ^{74}Se ceased after 6 weeks.

The results of our laboratory experiments showed that there was no significant variation in growth between juvenile *P. japonicus* fed a diet containing enriched levels of ^{74}Se and those fed the control diet (Fig 6). During the first six weeks the prawns grew from an initial mean wet weight 0.1 g to 0.4 g. In the subsequent six weeks, after feeding of the enriched diet had ceased, growth continued with prawns reaching a mean wet weight of 1.3 g after 12 weeks.

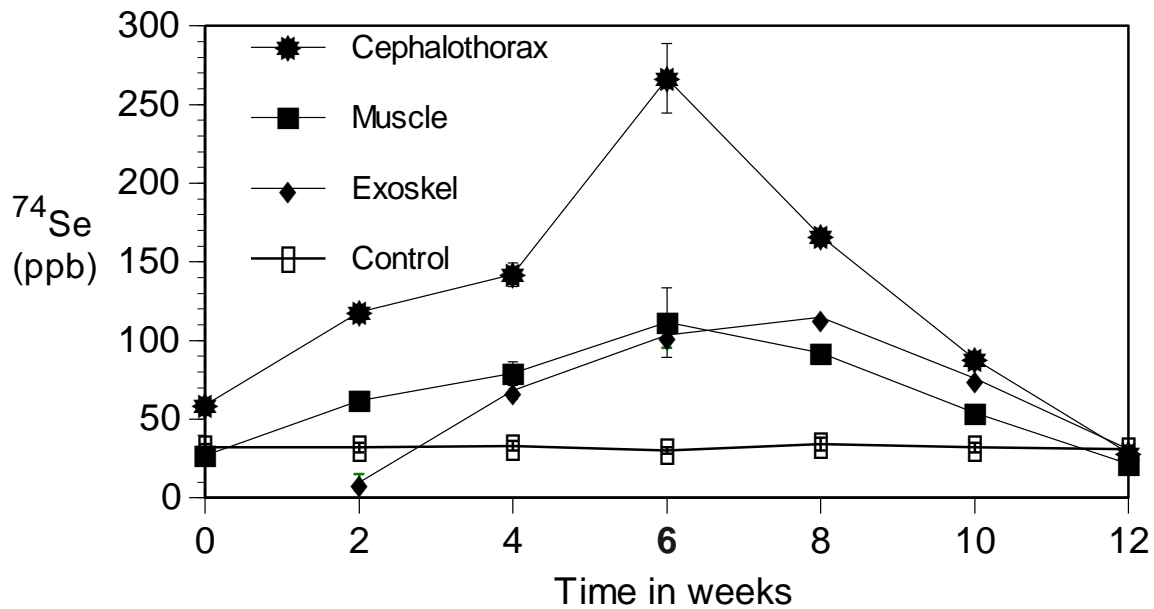


Figure 7. Variation in the concentration of ^{74}Se in the cephalothorax, abdominal muscle and carapace of juvenile *Penaeus japonicus* fed enriched ^{74}Se for six weeks followed by a control diet for six weeks.

During the six weeks when the juvenile *P. japonicus* were fed the diet with enriched ^{74}Se , the most rapid uptake and highest concentration of ^{74}Se was in the cephalothorax (Fig 7). Uptake of ^{74}Se was significantly lower in the abdominal muscle and carapace than in the cephalothorax. The levels of ^{74}Se in the carapace of animals sampled after two weeks was not significantly different to the initial concentration (the prawns moulted shortly before this sampling). There was no significant difference in the maximum concentration of ^{74}Se (100 ppb) in the abdominal muscle and carapace. When feeding of the diet enriched with ^{74}Se ceased the depletion in the cephalothorax mirrored the uptake rates, reducing to the starting concentration by the end of the experiment six weeks later. Two weeks and four

weeks after ^{74}Se feeding ceased the concentration of ^{74}Se was significantly lower in the abdominal muscle than in the carapace or cephalothorax but significantly higher than in prawns fed the control diet. On completion of the experiment, six weeks after feeding with ^{74}Se had ceased, there was no significant difference in ^{74}Se concentration between the any of the tissue types in the treatments or controls.

The uptake and residence time of ^{15}N and ^{13}C

In the laboratory experiments there was no significant variation in growth between juvenile *P. esculentus* fed the diet containing enriched levels of ^{15}N and ^{13}C and those fed the control diet (Fig 8). During the first six weeks the prawns grew from an initial mean wet weight 0.02 g to 0.15 g. In the subsequent six weeks, after feeding of the enriched diet had ceased, growth continued with prawns reaching a mean wet weight of 0.65 g after 12 weeks.

In juvenile *P. esculentus* that were fed a diet containing enriched levels of ^{15}N and ^{13}C for six weeks the maximum level of ^{15}N and ^{13}C in the abdominal muscle was attained after four weeks (Fig 9). Thereafter the level of ^{15}N and ^{13}C in the abdominal muscle declined.

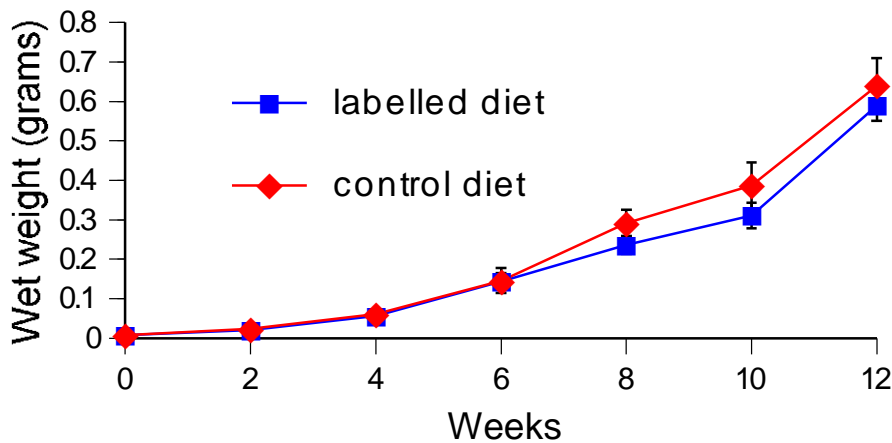


Figure 8. Comparison of growth rates of juvenile *Penaeus esculentus* fed enriched ^{13}C and ^{15}N for six weeks followed by a control diet for six weeks (labelled) and those fed a control diet

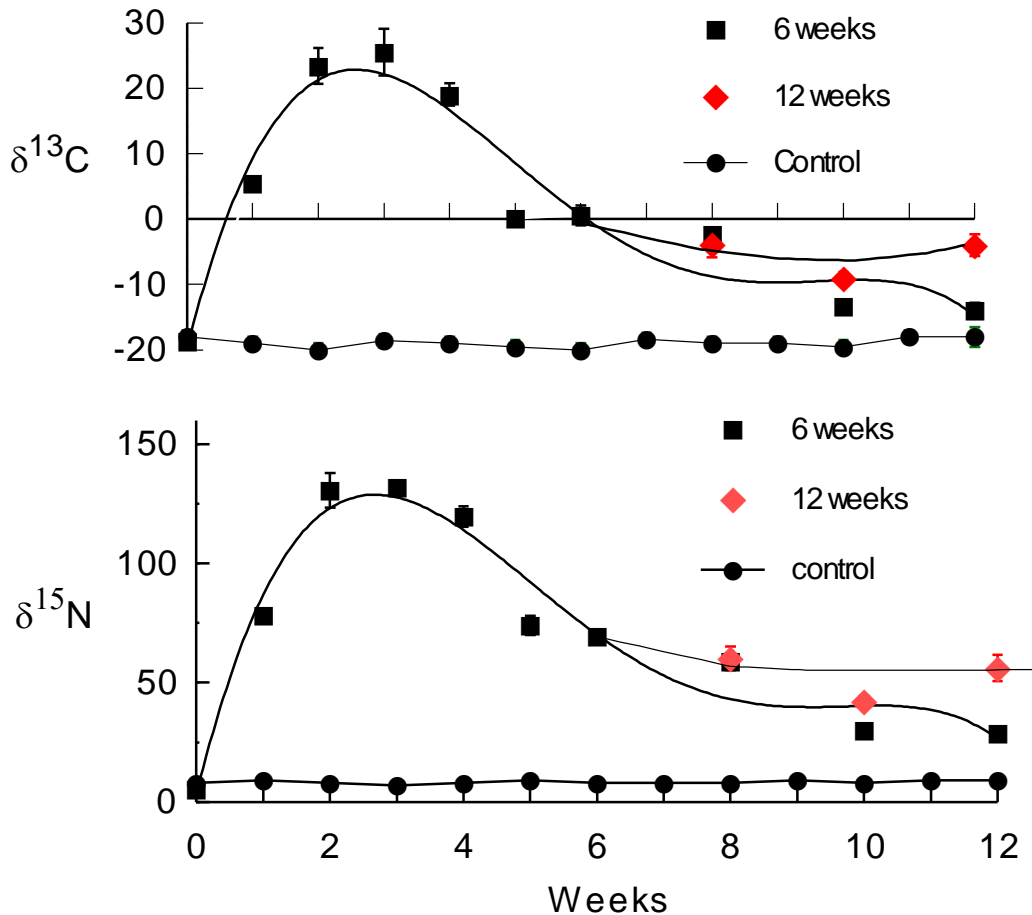


Figure 9. Variation in the concentration ^{13}C and ^{15}N in the abdominal muscle of juvenile *Penaeus esculentus* fed enriched ^{13}C and ^{15}N for six weeks or 12 weeks. Controls were fed on the same diet but without enriched ^{13}C and ^{15}N .

After 12 weeks the level of ^{13}C in the abdominal muscle of prawns that had been fed the enriched diet for six weeks was not significantly different from those reared on the control diet. However the ^{15}N level was significantly different to the levels in the controls. In the prawns that continued to feed on the enriched diet for the entire 12 weeks the levels of ^{15}N and ^{13}C were significantly different from the controls.

Discussion

The results of the laboratory experiments showed that a concentration of 5 ppm selenium is toxic to juvenile prawns, at least when this concentration is maintained in the diet over several weeks. This is consistent with toxicity levels in other marine invertebrates such

as amphipods and cumaceans (Ahsanullah & Palmer, 1980). Although we did not investigate the mechanisms controlling trace element concentration, the results of the pilot study (FRDC 92/07) and the laboratory experiments indicate that the mid-gut gland (located within the cephalothorax) plays an important role in this process.

The pilot study demonstrated that the trace element composition of the of juvenile prawns highly variable in the cephalothorax and less variable in the abdominal muscle. This is consistent with the laboratory results which showed that the levels enriched ^{74}Se were more highly variable in the cephalothorax than in the exoskeleton or abdominal muscle. Although the level of ^{74}Se in abdominal muscle progressively increased, in parallel with increased levels in the cephalothorax, the maximum concentration obtained in the abdominal muscle was 1 ppm compared with 2.5 ppb in the cephalothorax. This indicates a degree of regulation by the mid-gut gland. At the growth rates obtained in the laboratory the isotopic signature of the abdominal muscle persisted at detectable levels for four weeks.

The results obtained for ^{15}N and carbon (^{13}C) showed that these can also be rapidly elevated in small prawns via feeds containing highly enriched levels of the isotopes. The rate at which the rare isotopes are subsequently depleted will depend on the absolute level accumulated from the feed and the growth rate of the prawn post-release. Based on our results, we conservatively estimate that the carbon and nitrogen isotopic signatures of released juvenile prawn could be clearly separated from those of natural recruits for a period of 4-5 weeks post-release. Although the enriched ^{74}Se technique is equally effective we suggest that ^{15}N and ^{13}C would be the first choice in stock-enhancement trials because these are cheaper and easier to apply than ^{74}Se .

In summary this study has develop two novel types of biological tags for penaeid prawns: stable isotopes and genetic tags. We envisage these two types of tags being used for different purposes. The intended use of stable isotopes is as short-term chemical tags in pilot studies to monitor the fate of small prawns during the first few weeks after their release. If such trials were successful, genetic tags could then be used in subsequent

releases of genetically identified prawns. Genetic tags would also be required to monitor and maintain the genetic diversity of the enhanced populations.

Benefits

This project achieved its primary objective in providing novel, cost-effective methods of differentiating between introduced and wild prawns. These methods can now be used to monitor the effectiveness of prawn stock-enhancement trials and programs.

Apart from the successful development of novel biological tags for penaeid prawns this project resulted in the first successful captive breeding of Australian Kuruma prawns (*P. japonicus*). The captive-bred stocks were subsequently used to produce several generations of commercially farmed prawns (Preston *et al.* ^aunpublished). The same techniques could be applied to other native species including *P. esculentus*, that are under serious consideration for stock-enhancement.

Microsatellite DNA markers, similar to the *P. esculentus* markers isolated in this project, have subsequently proved to be effective for determining the paternity of prawns in laboratory selective breeding trials for *P. japonicus* (Moore *et al.* in press). The advances in domestication and tagging techniques developed in this project mean that it is now possible to produce and monitor chemically and genetically tagged prawns in stock-enhancement programs.

This project resulted in significant advances towards the development of gene transfer technology in penaeid prawns. However, although we successfully developed techniques for the introduction of reporter genes into penaeid prawns (Preston *et al.* ^bin press) we did not achieve stable transformation. Ultimately, genetic transformation will provide a powerful technique for examining the underlying biochemical and genetic processes of reproduction and defence against disease. The most significant application of this technology to penaeid prawns could be in genetically engineering traits such as improved growth or resistance viral diseases.

Intellectual Property and valuable information

The following information comprises the intellectual property arising from this project:

Chemical tags

- Techniques for introducing and accumulating elevated levels of a rare stable selenium isotope (^{74}Se) into the abdominal muscle of juvenile prawns
- Techniques for introducing and accumulating elevated levels of rare stable isotopes of nitrogen (^{15}N) and carbon (^{13}C) into the abdominal muscle of juvenile prawns
- Information about the effectiveness of (^{15}N), (^{13}C) and (^{74}Se) isotopic signatures of juvenile prawn for use as biological tags

Genetic tags

- Techniques for successful introduction of foreign DNA into prawn embryos via microinjection and electroporation
- Techniques for inducing transient expression of reporter genes in prawn embryos under the control of the fruit fly hsp70 and hsp82 heat-shock promoters
- Details of polymerase chain reaction screening of genomic DNA for *hobo*-like transposable elements giving positive results for *Penaeus esculentus*
- Techniques for the use of transposable elements as gene vectors in crustaceans as described in the provisional Australian patent (PN4593)
- The characterisation of microsatellite DNA markers in *P. esculentus* and mitochondrial DNA markers in *P. japonicus*

Further Development

It is anticipated that prawn re-seeding trials will soon commence in Australia. The application of the techniques developed in this project will be an integral component of any such program.

Research on the development of enabling gene transfer techniques for penaeid prawns is continuing within CSIRO.

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Final Cost

See separate financial report

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