Pilot study final report

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THE DEVELOPMENT OF BIOLOGICAL TAGS FOR PENAEID PRAWNS

PILOT STUDY FINAL REPORT

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THE DEVELOPMENT OF BIOLOGICAL TAGS FOR PENAEID PRAWNS

Report complied by: Nigel Preston

CSIRO Division of Fisheries Marine Laboratories
P.O. Box 120 Cleveland, Qld 4163 Australia
Phone (07) 286 8222 Fax (07) 286 2582

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EXECUTIVE SUMMARY

This project was a one year collaboration between the CSIRO Division of Fisheries, the CSIRO Division Entomology and the Queensland Department of Primary Industries (ICP-MS Laboratory, Animal Research Institute).

The purpose of the study was to determine the potential for developing novel biological methods of permanently tagging penaeid prawns at all stages of their life history.

The specific objectives were to:

- Determine the potential of gene transfer as a method of tagging penaeid prawns.
- Determine the potential of trace-element markers as a method of tagging penaeid prawns.
- Determine whether trace-element accumulation by juvenile prawns is specific to particular drainage basins.
- Determine the residence time of trace-elements accumulated by juvenile prawns

The main findings of the study were:

GENE TRANSFER

- Foreign DNA can be successfully inserted, transcribed and translated in prawn embryos.
- Gene transfer has the potential to provide a benign method of permanently tagging crustaceans at all stages of their life history.
- In addition to providing a unique genetic tag, the development of a genetic transformation system for penaeid prawns will provide the means to explore the potential of improved production through the genetic manipulation of farmed prawns

TRACE ELEMENTS

- The trace element composition of juvenile prawns differed significantly between locations and seasons (within locations) in the Gulf of Carpentaria.
- Variation in the trace element composition of diet or substrate significantly affect the trace element composition of juvenile prawns.
- Further controlled laboratory experiments are needed in order to determine whether prawns could be tagged using one or more trace-elements.

BACKGROUND AND JUSTIFICATION

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 tag has yet been developed for crustaceans 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 and there is currently no method for marking the post-larval or juvenile stages. 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 and Kirkwood, 1991). Frequent shedding of the exoskeleton precludes the use of the microchemistry of skeletal parts, other than for short periods. Prawns have a dispersive larval phase that ensures a degree of genetic mixing between regions thus limiting the use of natural genetic tags. Other limitations of natural genetic tags are the high costs of initial screening (to isolate and breed rare variants) and the high costs of distinguishing released animals from natural recruits.

Physical tags 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, harmless, 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, many of which have suffered from the lack of an effective tag (Farmer & Al-Attar 1981). Re-stocking programs have been most successful in China where the strategy of annual releases of 2 to 4 billion juvenile prawns into areas with relatively depleted natural stocks diminishes the need for a tag. However, in countries such as Japan, where the strategy has been to release farmed juveniles into areas with relatively large natural stocks, the re-stocking programs have been widely criticised for being very expensive and lacking a convincing method of assessing their cost/benefit.

In Australia natural stocks of prawns are relatively abundant but there is growing interest in the potential for re-stocking penaeid fisheries. Because of the relative abundance of natural stocks it would clearly be advantageous to have appropriate tagging technology to be able to distinguish released animals from natural recruits and thus evaluate the cost/benefits of any attempts at re-stocking. The objective of this pilot study was to assess the potential of two novel types of biological tags; reporter genes and trace elements, for permanently marking prawns of any size.

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. The specific objective of our pilot study was to determine whether the reporter genes used in *Drosophila* (fruit fly) are also expressed in prawns. If so, these reporter genes would provide precise, benign, biological tags for prawns. The rationale behind this research is the close relationship between penaeid prawns and terrestrial arthropods which offers the potential to take advantage of the prolific research into *Drosophila* genetics.

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, 1990). For example the hepatopancreas is a site of copper accumulation. The objective of this pilot study was to determine whether regional variations in the concentrations and relative abundances of trace elements in the sediments of prawn habitats are reflected in the composition of the hepatopancreas or other prawn tissues. We also sought to determine whether trace elements accumulated by *Penaeus merguiensis* (banana prawn) juveniles provide an imprint specific to particular drainage basins in the Gulf of Carpentaria. Ultimately, we wish to determine whether selected rare trace elements could be accumulated by captive juvenile prawns at sufficiently high concentrations to remain distinguishable in sub-adult or adult prawns, thus providing an effective biological tag.

METHODS

DNA MANIPULATIONS:

PRAWNS:

Penaeus japonicus were obtained from the wild (offshore from Mackay) or from a prawn Farm (Moreton Bay Prawn Farm) and stored in large tanks (approximately 15 animals in each 3m x 3m tank) at 27 °C , 35 ppt salinity. The prawns were fed frozen prawn and squid, and subjected to a reverse light cycle to ensure daytime spawning. Lights were switched on a 18.00 hrs and off at 06.00 hrs. Mature, fertilised females were ablated to promote spawning and placed in 50 litre spawning tanks. Following spawning, one-celled embryos were collected by pipette.

PLASMIDS :

Four plasmids used were:

- phspCAT - The bacterial chloramphenicol acetyl-transferase gene under the control of the *Drosophila melanogaster* hsp 70 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.

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.

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 $MgSO_4$ 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

Approximately 0.1nl of DNA was micro-injected into each one-celled embryo using a boro-silicate glass needle with an internal capillary tube. The needles were pulled to a fine point using a micropipette puller (Sutter Instruments - P87) with the following settings: Heat=450, Pull=90, Velocity=80 & Time=100. To facilitate the injection of multiple embryos, for each set of microinjections, approximately 30 to 40 embryos were sucked into a 10 cm length of silicate tubing with an internal diameter of 300 µm. The tubing was stuck to a glass microscope slide and injection port was cut into the side of the tubing. The

glass needle was carefully introduced through a thin layer of silicate tubing into each embryo. DNA was injected using low pressure compressed air controlled via a solenoid and foot switch. The embryos were moved along the silicate tubing by suction from a pipette.

ELECTROPORATION:

The electroporation apparatus (designed and built by CSIRO Division of Plant Industry, Canberra, Australia) delivered 1 pulse of 0-500V with settings of pulse lengths from 100μs 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.

The embryos were rinsed in sterile sea water, and up to 100 were removed in 400μl 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 create a kill curve to establish survival rates versus potential DNA entry, we investigated the effects of variation in electric field strength (V/cm) and pulse length (ms). Following the results of the kill curve (see results section) electroporation trials were done with two reporter gene systems, CAT and GUS. 10μg 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.

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 100µl staining solution (50µl 1M sodium phosphate, 20µl 100mM potassium ferricyanide, 20µl 100mM potassium ferrocyanide, 50µl 20mg/ml 5-Br-4-Cl-3-Indolyl-B-D-Glucuronidase (X-Glu) and 860µl 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. 50µl of the extract was reacted with 68µl 0.25M Tris-HCl Ph 8.0, 2ul ^{14}C-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 100µl 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 50µl 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 10µl 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.

TRACE ELEMENT SAMPLING AND ANALYSIS

FIELD EXPERIMENTS

PRAWNS:

Juvenile *Penaeus merguiensis* were collected from two major drainage basins in the Gulf of Carpentaria; Weipa and Karumba. In the spring of 1992 (november) samples of sediments and juvenile prawns were collected from 3 sites (replicates) at each of the locations (Weipa and Karumba). In the autumn of 1993 (march) the sampling of juvenile prawns was repeated for each location. Sediment samples were not collected in the autumn (due to the expense of sediment sample analysis). At each location the replicates consisted of pooled samples of 12 individual prawns (each approximately 1 gm in total weight). Pooling was necessary to obtain sufficient amounts of separate tissues (muscle, nerve, carapace and hepatopancreas) for trace element analysis. There were 3 replicate sediment cores at each location.

The prawns were collected during the day using a small beam trawl. Care was taken to ensure that the prawns did not come into contact with any metal. Following capture the prawns were held in aerated plastic tanks overnight (10 hours) to allow time for gut contents to be evacuated. The prawns were then frozen (-20 °C). Sediment cores were taken to a depth of 10 cm using a plastic coring tube. The cores were placed in plastic bags. On return to the laboratory at Weipa or Karumba the sediment samples were frozen. The frozen prawns and sediments were then transported by air to the Marine Laboratories at Cleveland.

Trace element analysis of sediment samples and prawn tissues was done by the Queensland Department of Primary Industries using inductively coupled plasma-source mass spectrometry.

In addition to the field studies, a laboratory experiment was done to determine the effects of diet and substrate on the trace element composition of juvenile prawns.

LABORATORY EXPERIMENT

The prawns used in the laboratory experiment were juvenile *Penaeus monodon,* obtained from Gold Coast Marine Prawn Farm, situated at the mouth of the Logan River. In the experimental design there were two diets (CSIRO diet formulation and a commercial diet formulation) and two substrates (black sand and white sand). There were two replicates in each treatment. Each replicate comprised 10 juvenile prawns each approximately 2 grams total weight.

The animals used in the experiment were maintained in fibre glass tanks (60 x 60 x 40 cm) lined with 4 cm of substrate. The animals were fed twice daily (08.00 and 18.00). The pelleted feeds were presented on feed trays and the uneaten remains of pellets were removed and weighed each day. The experiment was run for 2 weeks. Animals collected from the farm at the start and end of the experiment served as controls. On completion of the experiment the animals were frozen and sent to the ICPMS lab for trace element analysis together with samples of the two diets and two substrates.

RESULTS

REPORTER GENES

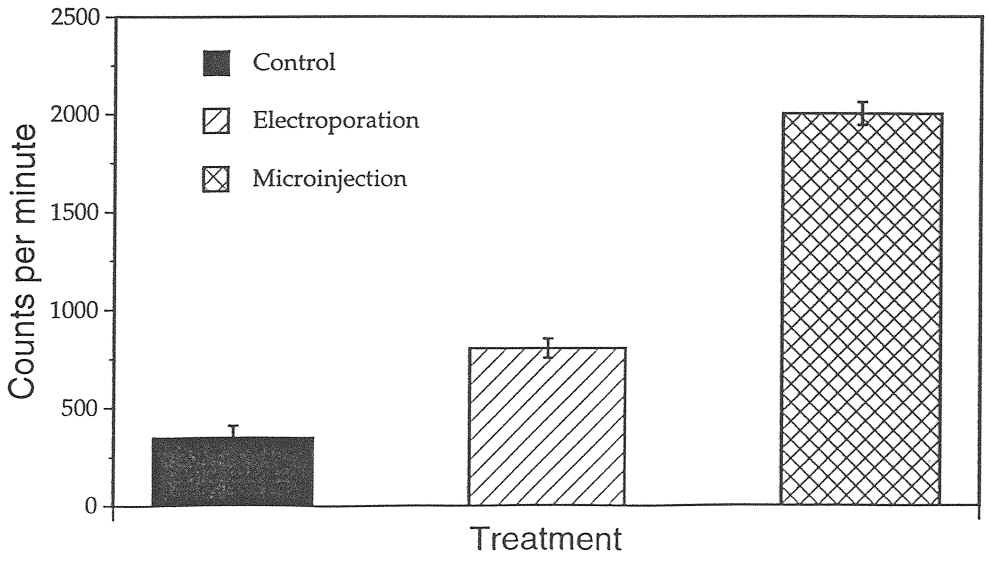
In this pilot study we examined the performance of four reporter gene/plasmid constucts following microinjection into one-cell stage embryos of *Penaeus japonicus*. We used the same reporter gene/plasmid constucts to explore the potential inserting DNA into prawn embryos using electroporation.

MICROINJECTION

The technique that we developed for micro-injecting prawn embryos allowed us to successfully inject batches of 30 to 40 embryos before the first cell division. In the course of the study we injected approximately 800 one-celled *Penaeus japonicus* embryos. These comprised approximately 100 embryos for each of the four plasmid constructs and approximately 400 controls injected with seawater without DNA.

The results of the enzyme assays gave positive results for one of the reporter genes: pHspCAT A under the control of the *Drosophila melanogaster* hsp70 promoter (Fig. 1). None of the other 3 plasmids showed any positive signs of enzyme activity following microinjection into prawn embryos.

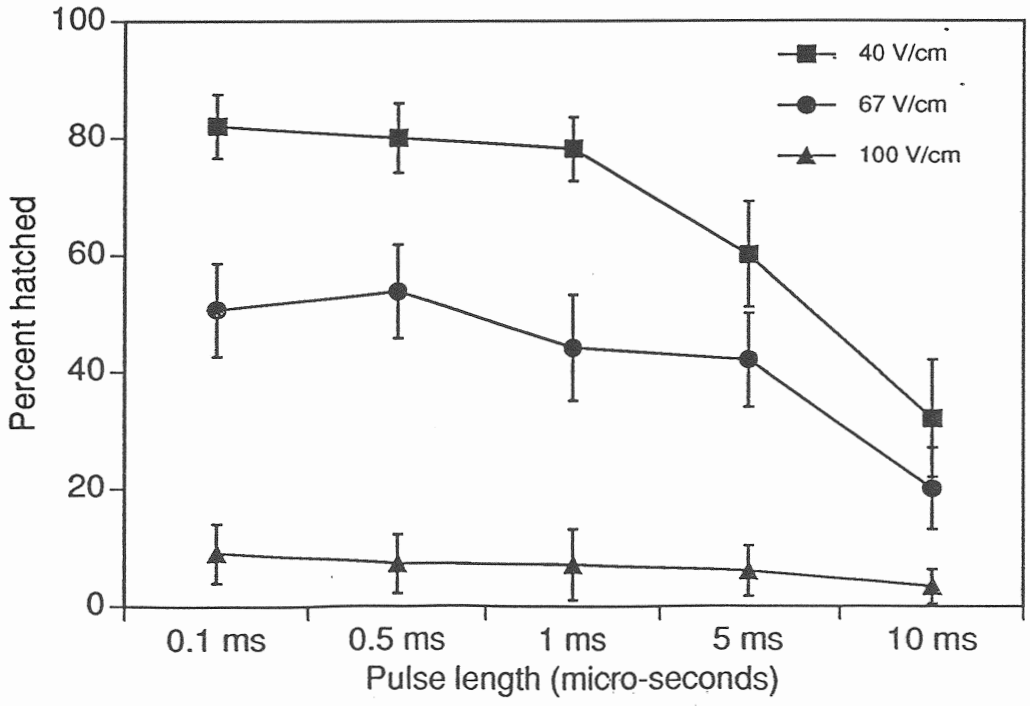
Figure 1. Scintillation counts of the levels of Chloramphenicol acetyl transferase (CAT) gene products in *Penaeus japonicus* embryos following electroporation or microinjection of pHspCAT A under the control of the *Drosophila melanogaster* hsp70 promoter.



ELECTROPORATION

The results demonstrated that the hatching success of embryos varies according to pulse strength and pulse duration (Fig. 2). Pulse strength had a more pronounced affect than pulse length, particularly for pulse lengths of less than 1 ms. The highest hatch rates were obtained at the lowest pulse strength (40 V/cm) and the lowest hatch rates at the highest pulse strength (100 V/cm) minimum. These preliminary data indicate that the intermediary hatch rates, obtained with a pulse strength of 67 v/cm and a pulse length of 1 ms, provided appriopriate conditions for subsequently testing electroporation as a method of DNA delivery. Accordingly we tested for CAT and GUS gene expression following electroporation. No positive results were obtained for GUS expression. Positive results were obtained for CAT expression (Fig. 1). However, the relatively low level of gene expression indicated that DNA delivery was less efficient than with micro-injection.

Figure 2. The effects of electroporation pulse strength (V/cm) and pulse length (ms) on the hatch rates of one-celled *Penaeus japonicus* embryos.



TRACE ELEMENTS

For each of the prawn tissue types for both locations (Weipa and Karumba) and for both seasons (spring and autumn) the concentration of 35 trace elements was determined. For each of the sediment samples, from both locations, the concentration of 49 trace elements was determined. Following the ICPMS results the trace elements were assigned to the following categories:

PRAWNS

- Major physiological elements (or paired with major physiological elements) in penaeid prawns. The elements in this category were: Na, Mg, S, P, Ca/Sr, Cu/Zn. Total=8. These elements had no potential as a tag because they vary according to physiological needs. The results for these elements were not analysed any further.

- Elements with concentrations in tissues below the level of reliable detection by ICPMS (>0.01 ppm). The elements in this category were: Rh, Bi, Ho, Tb, Tm, Lu, Tl and Y. Total=8. The results for these elements were not analysed any further.

- Essential trace elements found at concentrations in prawn tissues above the level of reliable detection. The elements in this catergory were: Fe, Mn, Cr, V, Mo, Co, Se, As and Ni. Total=9. The concentrations of these elements were examined in relation to to tissue type, location and season using pattern analysis and/or anaysis of variance.

- Non-essential trace elements found at concentrations in prawn tissues above the level of reliable detection. The elements in this catergory were: Ag, Al, B, Cs, Ce, U, Pb, Li, Ba and Cd. Total=10. The concentrations of these elements were examined in relation to to tissue type, location and season using pattern analysis and/or anaysis of variance.

SEDIMENTS

- Elements measured in both sediments and prawn tissues at found at levels above the level of reliable detection, and which were not controlled physiologically. The elements in this category were:
  Fe, Mn, Cr, V, Mo, Co, As and Ni
  Cs, Ce, U, Pb, Li, Ba and Cd

The relative abundance of the essential and non essential trace elements are shown in Figures 3 & 4 on a $\log_{10}$ scale of abundance according to tissue type, location and season. These figures show that the concentration of trace elements was highly variable among tissue types. Generally, trace elements were found at higher concentrations in the hepatopancreas than in other tissue types. Conversely, the tail muscle appeared to be the most conservative tissue type in retaining trace elements.

Comparison of the relative abundances of trace elements between locations and seasons, within each tissue type, indicated greater variation in the trace element composition of prawn tissues between seasons than between locations (Figs 3 & 4).

Analysis of variance for each tissue type and each trace element demonstrated significant variation in the concentration of some trace elements due to location and/or season (Table 1). The source of variation varied among the tissue types. For example, in the hepatopancreas 14 of the 20 elements analysed showed significant variation between seasons but only 6 of the 20 elements showed significant variation between locations. In contrast to this, analysis of tail muscle showed significant seasonal variation in 6 of the 20 elements and significant variation in 6 out of 20 elements due to location.

Analysis of variance demonstrated significant differences between Weipa and Karumba sediments in the concentration of all 15 trace elements, in at least one size fraction (Table 1).

Figure 3. The relative concentrations (log_{10}) of essential trace elements found in in tissues of juvenile banana prawns collected from Karumba and Weipa in spring and autumn.

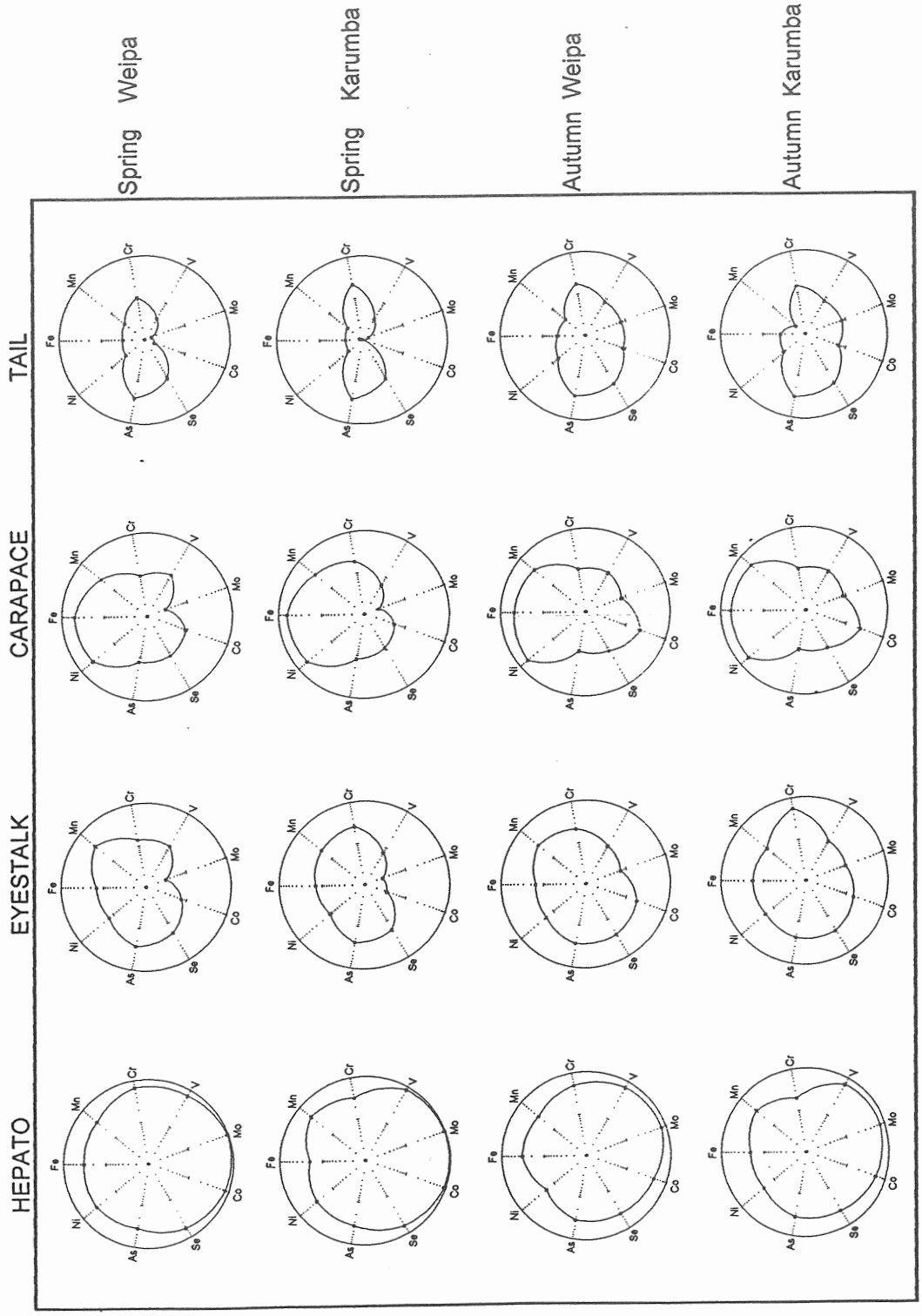


Figure 4 The relative concentrations (log_{10}) of non-essential trace elements found in in tissues of juvenile banana prawns collected from Karumba and Weipa in spring and autumn.

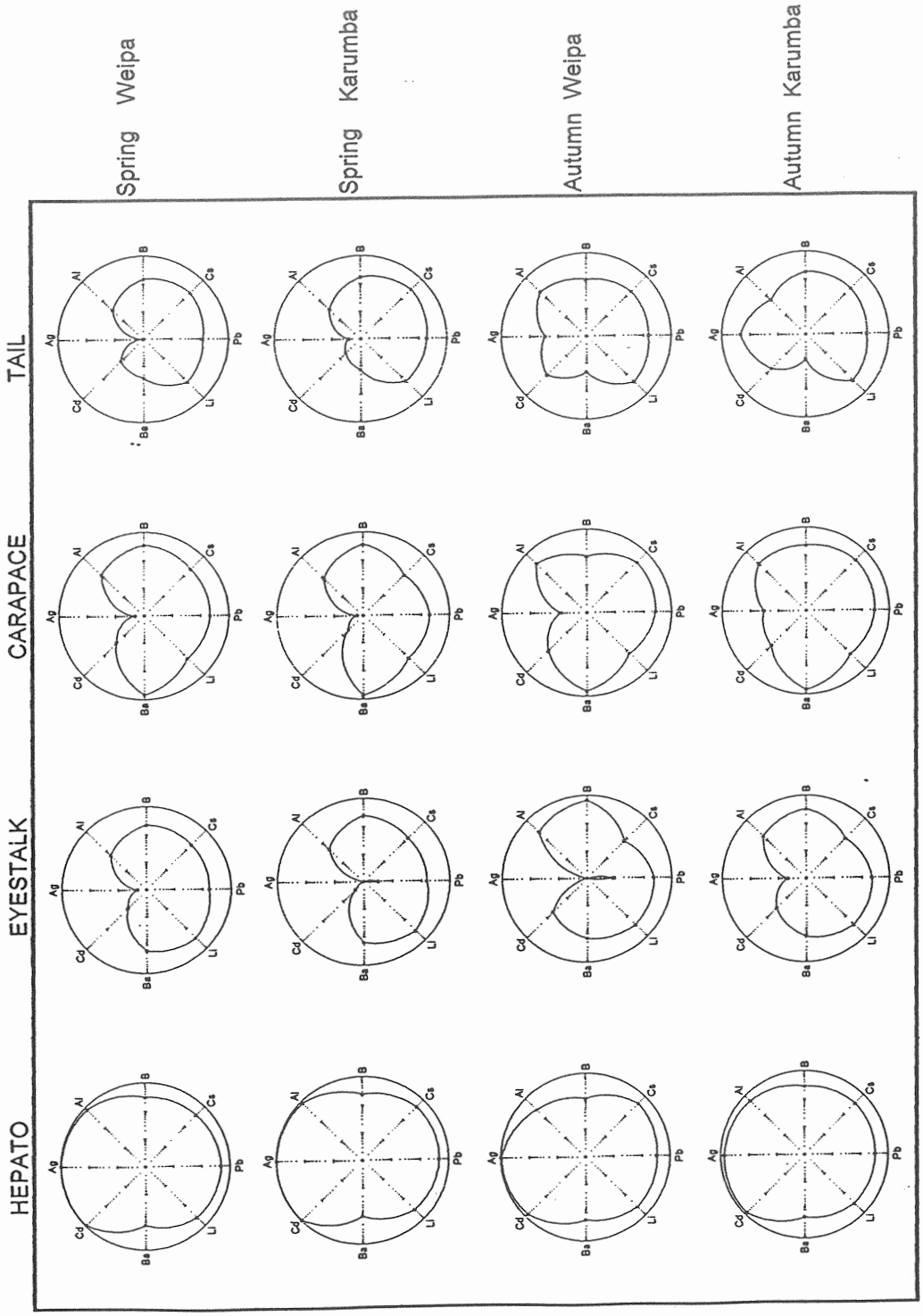


Table 1. Analysis of variance of the concentration of trace elements in the hepatopancreas, eyestalk, carapace and tail muscle of juvenile prawns collected from Karumba and Weipa in spring (november) and autumn (march). Analysis of variance of the concentration of trace elements of three size fractions of sediments (2.0 - 0.2 mm, 0.2 - 0.063 mm and <0.063mm) collected from Karumba and Weipa in spring.

						Trace elements													
Source of variation	Ag	Al	As	B	Ba	Cd	Ce	Co	Cr	Cs	Fe	Li	Mn	Mo	Ni	Pb	Se	U	V
Hepatopancreas																			
Time	***	*	ns	**	*	**	ns	*	ns	**	ns	***	ns	**	ns	**	**	**	*
Place	**	ns	ns	ns	**	**	ns	ns	ns	*	ns	ns	*	ns	ns	ns	ns	*	ns
Time x Place	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Eyestalk																			
Time	ns	*	ns	ns	ns	***	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns
Place	**	ns	ns	ns	***	**	ns	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns	ns
Time x Place	**	ns	ns	ns	ns	**	ns	ns	ns	ns	*	**	ns	ns	*	ns	*	ns	ns
Carapace																			
Time	ns	ns	**	***	ns	*	ns	ns	ns	**	ns	***	ns	ns	ns	ns	*	ns	ns
Place	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	*	**	ns	*	**	ns	ns	ns	ns
Time x Place	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns
Tail muscle																			
Time	ns	ns	ns	*	ns	***	ns	ns	ns	ns	ns	***	ns	***	ns	***	**	ns	ns
Place	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	**	**	*	ns	ns	*	ns	ns
Time x Place	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Sediment																			
a: 2.0 mm - 0.2 mm	-	-	***	-	***	ns	ns	*	ns	ns	**	ns	ns	***	ns	ns	-	***	***
b: 0.2 mm - 0.063 mm	-	-	*	-	***	ns	*	ns	ns	ns	ns	ns	**	***	ns	ns	-	ns	*
c: <0.063 mm	-	-	***	-	***	**	ns	***	***	ns	**	ns	***	***	***	***	-	ns	***

LABORATORY EXPERIMENT

Preliminary results of the laboratory experiment demonstrated that the trace element composition of juvenile prawns varied in response to diet and substrate (Figs. 5 & 6). The results indicate that the influence of diet is more pronounced than that of substrate and that the degree of influence varies with tissue type. For example cadmium levels were significantly higher in the hepatopancreas of animals fed diet B (commercial formulation) than those fed diet A (CSIRO formulation). However, there were no significant differences in the tail muscle cadmium levels among the different diets and substrates. Similar results were obtained for lithium, an element that appears to be conserved in tail muscle muscle (Fig 4).

Figure 5. The effects of diet (diets A & B) and substrate (ws = white sand, bs = black sand) on cadmium concentration in hepatopancreas and tail muscle of juvenile banana prawns

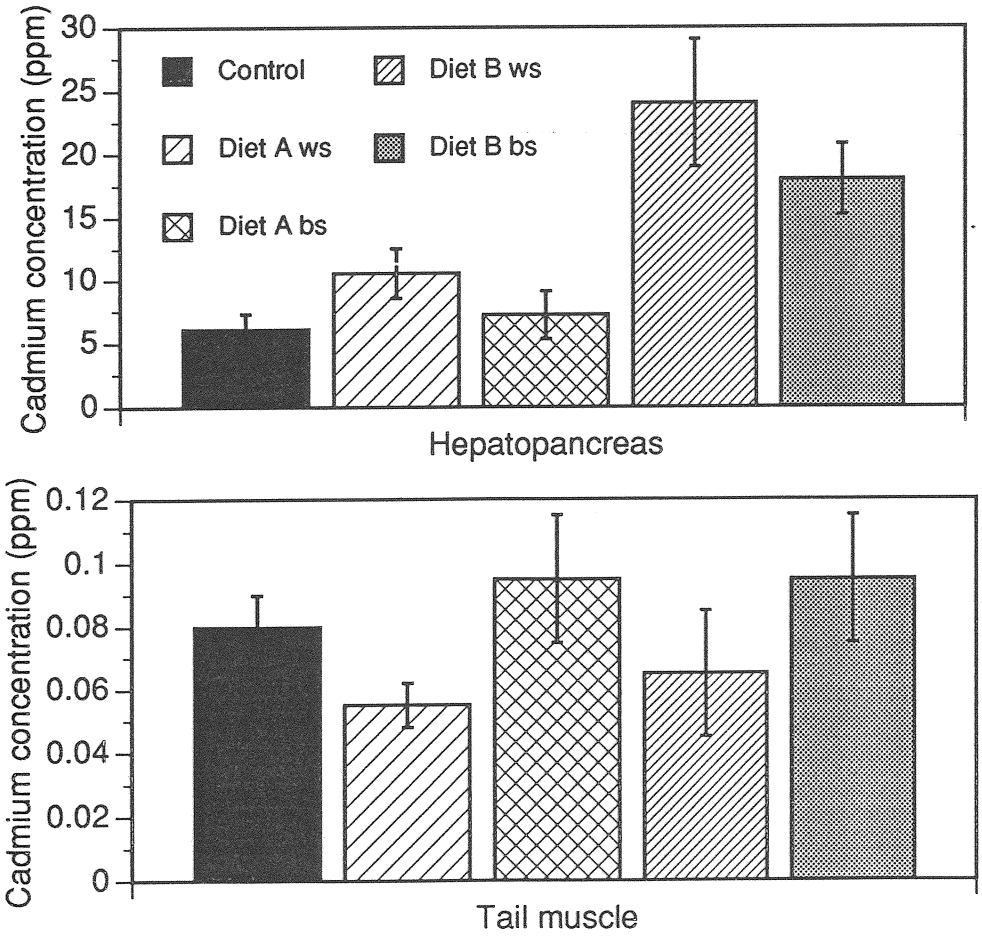
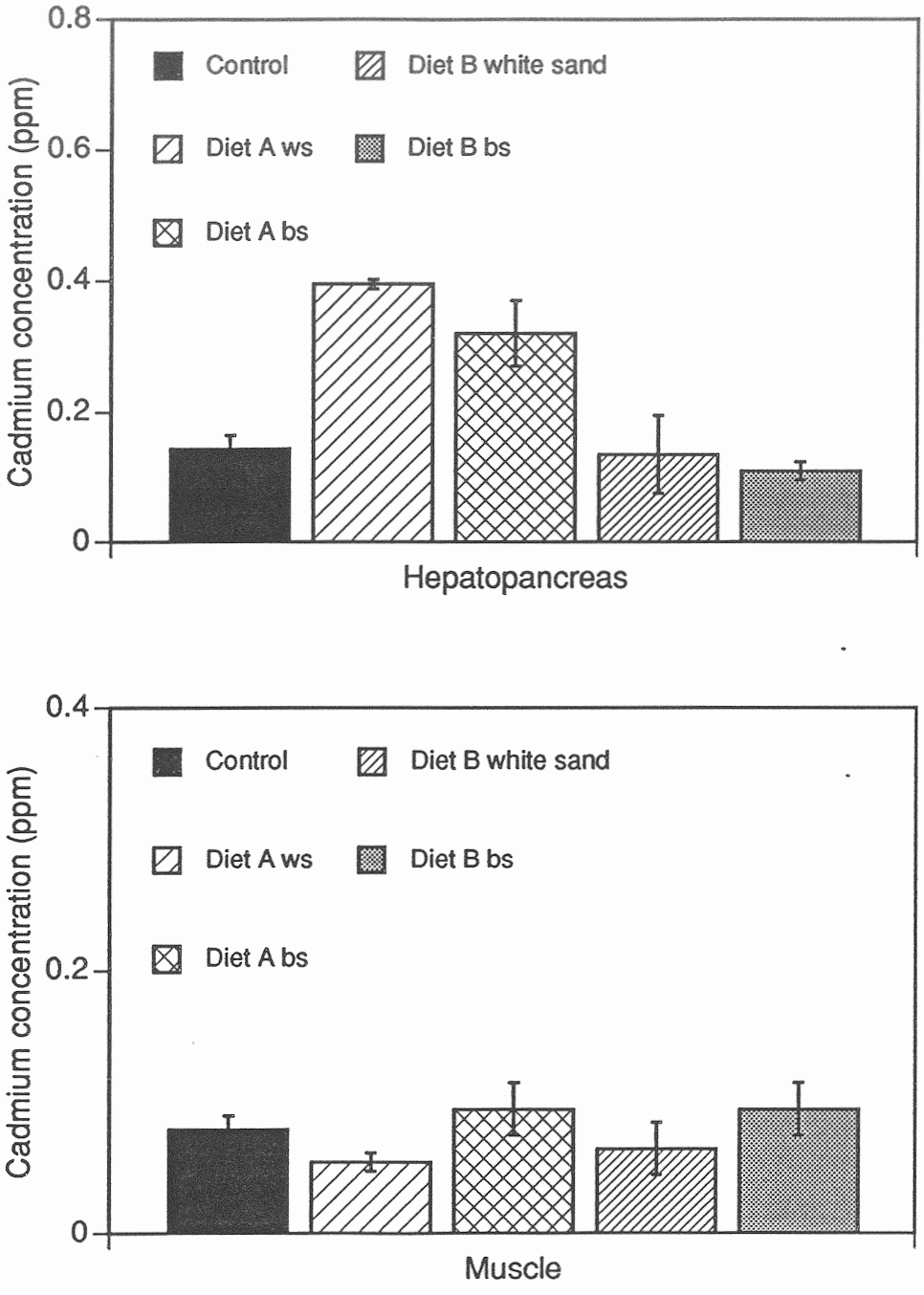


Figure 5. The effects of diet (diets A & B) and substrate (ws = white sand, bs = black sand) on lithium concentration in hepatopancreas and tail muscle of juvenile banana prawns



DISCUSSION

The results of this pilot study indicate that reporter genes and trace-elements do have potential as biological tags but both techniques require further investigation and development.

REPORTER GENES

We succeeded in micro-injecting a reporter gene (phsp-CAT under the control of the *Drosophila melanogaster* hsp70 promoter) into prawn embryos and, subsequently, detected the production of the introduced enzyme within embryos. This is the first report of gene transfer in penaeid prawns. It is an important step towards developing a gene transformation system for penaeid prawns (i.e. adding novel, inheritable foreign genes). Apart from providing a tag, gene transfer would also provide a powerful tool for exploring the potential of improving growth rates or disease resistance in farmed prawns through genetic manipulation. To capitalise on the success of our pilot study we now need to develop an efficient and reliable method of gene transfer in prawns.

The results of this study demonstrate that penaeid prawn embryos can withstand the effects of electroporation. Survival rates of approximately 30% are considered optimal in electroporated mammalian cells (Knutsen & Yee, 1987). Within the set of electroporation conditions that we tested we were able to obtain hatch rates of approximately 40% with a pulse length of 1 ms and a pulse strength of 67 V/cm. However, our preliminary trials with reporter genes indicate that DNA delivery was less efficient than with micro-injection. The most likely explanation for this is the low permeability of the hatching membrane. Total or partial removal of the hatching membrane with 3 amino-triazole (Lynn *et. al.*, 1993) should permit more efficient delivery of DNA.

TRACE ELEMENTS

The results of our pilot study have demonstrated pronounced differences in the trace element composition of juvenile prawns collected from two widely separated drainage basins in the Gulf of Carpentaria. However, the results

have also shown pronounced temporal variation in trace element composition of juvenile prawns within locations. From these results it seems unlikely that any suite of natural trace elements accumulated by juvenile prawns would remain distinguishable in offshore adult populations.

Preliminary analysis of the results of our laboratory experiments provided evidence that the trace element composition of the food eaten by of juvenile prawn has a pronounced effect on the trace element composition of the hepatopancreas (mid-gut gland) but less of an effect on abdominal muscle. The influence of the trace element composition of sediments (substrate) appears to be less pronounced than the influence of the trace element composition of the diet of juvenile prawns. Further analysis is being done to partition the relative effects of diet and substrate.

Despite the level of variation observed in the trace element composition of juvenile prawns, the question remains whether the trace element composition of prawns could 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 muscle or nerves, and later be detectable in the sub-adults and adults.

Comparison of the different prawn tissue types examined in this study revealed that tail muscle generally contained lower concentrations of most trace elements than other tissue types. The difficulty of obtaining nervous tissue from small prawns and the relatively conservative trace element composition of tail muscle indicate that tail muscle may be a more appropriate target tissue.

Among the trace elements that are considered essential for prawns, arsenic and selenium both accumulate in tail muscle. Among the non-essential elements lithium, lead, caesium and boron all accumulate in tail muscle. The relatively high natural abundance of all these elements preclude their use as a tag (released animals could not be distinguished from natural recruits). One option could be to use, where possible, enriched stable isotopes of these

elements (for example selenium contains six stable isotopes). These isotopes are expensive but, depending on tissue retention, could still be cost effective. The uptake and retention of an enriched selenium isotope should be investigated.

Apart from using enriched isotopes of trace elements, two alternatives remain; the use of very rare trace elements such as iridium or europium or the use of highly enriched stable isotopes of very common elements such as carbon, nitrogen or sulphur. The very rare elements are prohibitively expensive and of unknown toxicity. The use of highly enriched isotopes of common elements could provide an effective tag and is worth investigating.

FURTHER STUDIES

The need for further investigations of novel types of biological tags should be viewed in the context of their proposed application. Implicit in the purpose of developing new techniques for tagging prawns is the concept that re-stocking penaeid fisheries will be trailed in an existing Australian prawn fishery. This assumes that advances in prawn aquaculture in Australia will make it possible to produce sufficient numbers of juveniles at very low cost. If attempts at re-stocking are made, the ability to administer and detect an effective tag at low cost would be of considerable benefit.

In assessing the attributes of an ideal tag for penaeid prawns Rothlisberg and Preston (1991) considered that altered genetic tags (reporter genes) or chemical tags (trace elements) would have a greater number of positive attributes than natural genetic or physical tags. The disadvantages of physical tags are that they cannot be used on larval, post-larval or early juvenile stages. They are also expensive and labour intensive to apply. The primary disadvantage of natural genetic tags is that they would be expensive to establish and monitor. In the first instance the natural population would have to be screened for rare variants. Providing one or more variants were detected large numbers of animals would then have to be bred in captivity from a small number of parents. Following release and recapture the screening of large numbers of animals for natural genetic tags would be expensive at current prices. It is,

however, conceivable that more rapid, lower cost screening methods could be developed in the future.

Altered genetic markers (reporter genes) would not require initial screening but would probably require a captive breeding program (as opposed to directly tagging the progeny of wild stocks). Altered genetic markers have the potential of providing multiple markers and very rapid and cheap detection.

The type of chemical tag required is one that could be administered internally in very low doses at an early stage, be retained throughout life in conservative tissues (such as nervous tissue) and be cheap to detect.

The primary reason for continuing the development of novel tags for prawns is to substantially reduce the costs of tag application and detection. Conventional tagging methods (physical and genetic) are currently too expensive to be seriously considered as effective tools for monitoring the cost-benefits of commercial scale re-stocking attempts.

Planned publications from this project:

Atkinson, P.A., Preston, N.P., Henderling J (in prep). Applications and constraints to genetic manipulation of marine invertebrates

Preston, N.P., Die, D.J., Courtney, A.J., Austin M. (in prep). Trace element compostion of juvenile prawns from different habitats.

Die, D.J.,Preston N.P., Courtney, A.J., Austin M (in prep). The realtive influences of diet, substrate and water on trace element accumulation by juvenile penaeid prawns.

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