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FISHERIES RESEARCH & DEVELOPMENT CORPORATION

2011/245 Tactical Research Fund:

Research methods to manage pathogenic microbiological and biological organism within a redclaw (*Cherax quadricarinatus*) egg incubator hatchery to improve survival and reliability

JAMES COOK UNIVERSITY
AUSTRALIA

AquaVerde
Redclaw Hatchery & Farm

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Date: June 2013

Publisher: AquaVerde Redclaw Hatchery & Farm

ISBN: 978-0-646-90429-0

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1. Non-Technical Summary

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OBJECTIVES:

1. Bacterial & fungal identification & management
2. Test commercial probiotics / develop in-house probiotics and best practice
3. Determine critical time when hatched larvae need to start feeding
4. Identify causes for unexplained mortalities at all life stages (egg, larvae, crayling)
5. Develop methods to identify viable/unviable eggs

OUTCOMES ACHIEVED TO DATE

Production in the hatchery has already significantly improved due to a greater understanding of the microbiological implications researched in this project. This is demonstrated in better management of the micro flora / fauna within the egg incubators, which leads to higher consistency in survival of the craylings and reliability of the system.

The extra hatchery production has been immediately taken up by redclaw growers with increasing demand for next season. The outcomes so far:

Farm husbandry

- Increased yields and extended season for crayling production
- Increased profitability for growers by freeing them up from using brood stock / juvenile ponds
- Improved efficiency by simplifying production, reducing duplication and work load on the farmer
- For the first time farmers are able to stock exact quantities of same age craylings and so will be better able to predict growth rates and bio-mass, to better manage feeding rates / water exchange and predict production
- Facilitate the move from an extensive to a more intensive aquaculture industry
- Earlier stocking of craylings in spring to increase summer grow-out period

Re-vitalising a stalled industry.

- Through our growing knowledge and expertise we can provide more guidance and consultation to growers
- New entrants to the industry can save the time it would take to grow their own brood stock and can have an immediate start up with hatchery produced craylings.
- Increasing production (more profitable for producers)
- Farmers report reduced work load

Better control of the lifecycle of redclaw crayfish opens new areas of research

- This technology has already allowed the North QLD Crayfish Farmers Association (NQCFA) to implement a selective breeding project that would have been far less efficient (with less control over family lineage) if traditional crayling production methods were used. As we have obtained a high level of confidence AquaVerde has now taken over the selective breeding project with the incubation technique being the pivotal part of the process.

It has been identified by the Queensland Crayfish Farmers Assoc. (QCFA) and acknowledged by DAFF (Fisheries Queensland) that for our redclaw industry to grow and develop beyond "extensive farming practices" we require a reliable hatchery for producing disease free seed stock all year round, which will lead to radical changes in farm husbandry which in turn increases production and supports the re-vitalisation of a stalled industry.

AquaVerde secured a crayfish egg incubation system that was originally developed in Scandinavia for disease mitigation (producing crayfish populations free of *Aphanomyces astaci*). It became clear that we could use such an incubator with redclaw to provide the basis for a hatchery. The system basically works by harvesting the fertilised eggs from females and incubating them in baskets in a controlled aquatic environment. Within the baskets, the eggs hatch and progress through two larval stages before they finally become morphologically adults, termed Stage 3 Juveniles (S3J's) and what we also refer to as "craylings". These techniques afford an unprecedented level of control over the lifecycle of freshwater crayfish and have many advantages over the old traditional methods of "just throwing brood stock into ponds and seeing what you get after 12 months".

Good progress was made with the hatchery but microbiological infections occasionally caused high mortalities in predominantly Stage 2 Larvae (S2L) that we were unable to solve. It became clear that to build a reliable hatchery for our industry we needed expert help. Support from leading figures in aquaculture encouraged us to apply for this FRDC Tactical

Research Fund to engage microbiologists for research. We found just the people we needed at the School of Veterinary and Biomedical Sciences, James Cook University Townville.
The main priority was to identify pathogenic bacteria and find ways to manage them.
The use of probiotics has worked well in various aquaculture facilities around the world and most notably in prawn hatcheries. Therefore our main focus was to find and culture suitable non pathogenic bacteria that could be used as a probiotic(s) to competitively exclude pathogens to sublethal dose. The best place to look for suitable probiotic bacteria is within the system itself. A regime of sampling from the hatchery began to identify the pathogenic bacteria and at the same time to identify suitable candidate bacteria for use as a probiotic.

During the studies the main culprit was identified as *Aeromonas hydrophila*. An exception was the first isolation, when *Chromobacterium* species was found to be the cause of disease. Many other bacteria were isolated and some selected to test their suitability as a probiotic. All the candidate probiotic bacteria selected so far have failed to completely contain *A. hydrophila* when tested under laboratory conditions. However we will still trial them in the hatchery to evaluate if they are able to inhibit *A hydrophila* below lethal levels for long enough to allow Stage 2 Larvae to reach Stage 3 Juveniles, at which point they become better able to resist infections.

The use of a commercially available probiotic containing *Bacillus* species was shown not to inhibit the development of the *A. hydrophila*-associated bacterial septicaemic disease in the S2L's. Nor did they appear to inhibit the proliferation and biofilm formation of *Aeromonas hydophila* in the water. A laboratory study showed that the *Bacillus* species were not able to inhibit the growth of both *Chromobacterium*, which was responsible for a single outbreak of disease, and the more common *Aeromonas hydrophila*. Although the probiotic bacteria were able to result in the formation of early biofilms in the hatching tank after cleaning, *Aeromonas hydrophila*, once introduced, was able to out-compete the *Bacillus* species. This was in spite of the fact that the dose and rate of administration of the probiotic *Bacillus* species had been higher than the standard recommended dosages.

The ongoing cultures and histological examination of both healthy and affected eggs and larvae enabled us to associate the bacteria cultured with those observed histologically and infer the causative agent of the mortalities, especially in the S2L. Histological examination of the larvae also allowed us to detect whether any viral inclusions were present which may have contributed to disease or increasing the susceptibility of the larvae to opportunistic

bacterial infections. In this study, none were observed. Furthermore, histological examination of healthy larvae was able to show that the S2L's had a higher number of bacteria within their intestinal tract and hepatopancreas than the S3J's.

The evidence of an infection by bacteria common in freshwater that enter after hatching and affected predominantly the hepatopancreas of the larvae indicated the necessity of at least attempting to control the most obvious source of the bacteria. This directly led to changes in hatchery management regarding better control of the microbiological quality of the incoming water and the control of aerosolised water droplet formation by reducing relative humidity of air within the hatchery from over 90% to just under 50%.

We also performed feeding trials as part of this research project to try to determine the critical stages when S3J's (craylings) need to be fed to survive their first post juvenile moult. Previous studies on the feeding of S3J's have always begun at the point the craylings were released from the mother's tail, which is not entirely relevant in our situation. Because of this we felt the need to begin our own experiments. We set up nine small tanks with three feeding regimes (no feed, prawn larvae dust, and blood worms, replicated 3 times) and ran the trials three times each with S3J's craylings of different ages (2, 7, 14 day old). The S3J's were taken from the incubator and stocked into the 9 tanks and feeding began to determine if the time held without feeding in the incubator affected survival till first post juvenile moult. The survivability of redclaw craylings was much higher when fed than when they were not fed. Also, the results indicated that S3J's should be fed, but it may not be necessary to feed them before they are 14 days of age as demonstrated in the feeding trials.

KEYWORDS: redclaw crayling, hatchery, incubation, microbiology, bacteria

2. Acknowledgements

This project is supported by funding from FRDC on behalf of the Australian Government. It is encouraging that not only pure systematic research in laboratories is funded but also research and observational problem solving in an on-farm environment.

Thanks to James Cook University Townville for its generous in-kind contributions and especially to Dr. Jackie Picard for her advice and guidance. Associate Professor Leigh Owens was very generous with his time offering valuable direction. Many insights were provided by these researchers, demonstrating a desire to assist in developing a strong redclaw industry.

Thanks to industry leaders John Stevenson (president Queensland Crayfish Farmers Association) and Dr Trevor Anderson (former president of the Queensland Aquaculture Industries Federation) and General Manager of Seafarm, Cardwell. Their encouragement and commitment to improving invertebrate aquaculture and their belief in the importance of developing a hatchery as a foundation for a successful redclaw industry has been inspiring. Many thanks also to Ian Anderson (principal veterinary pathologist fish disease, DAFF QLD) for pathology work, advice and his support for this project.

Special thanks to Max Wingfield DAFF QLD, Bribe Island for his unwavering dedication to redclaw.

And finally to all the redclaw farmers in Queensland who have supported us in so many ways. Thank you for making your farms available for trialling hatchery produced craylings or for providing a regular supply of eggs. Your feedback and belief in building an industry based on incubation technology has kept us going these last five years.

Photographs courtesy of AquaVerde Redclaw and JCU

3. Background

Already in 1995 it was recognised by Dr. Clive Jones, that "as the (redclaw) industry develops, the demand for quality juveniles is likely to increase. To meet this demand, specific juvenile production technologies will be required, which may involve selective breeding programs to maximise optimal traits" (Jones, 1995).

Although the hatching of viable redclaw eggs in incubators has been observed to be above current industrial norms, the survival of larvae, especially Stage 2 Larvae (S2L) to the first adult moult is highly variable. Preliminary studies indicate that a possible reason for the poor survival of larvae is septicaemia resulting from an *Aeromonas hydrophila* infection. *Aeromonas hydrophila* is common in tropical freshwater bodies and readily colonises the intestinal tracts of freshwater reptiles and amphibians, fish and crustaceans. Occasionally virulent strains of this bacterium will cause skin and systemic disease in these species. This usually occurs in stressed animals, those with damaged skin or when they are found in very high numbers (Jiravanichpaisal *et al*., 2009). However, other bacteria and viruses are known to cause disease in redclaw crayfish (*Cherax quadricarinatus*) (Edgerton *et al.*, 1995; Edgerton *et al.*, 2000).

The redclaw hatchery has at times experienced high mortalities of especially S2L. The cause/s of these is still not known. Therefore, it is important to determine what the cause/s of these mortalities are and to identify the factors that may contribute to the most common cause/s of disease. Antibiotics, and in particular oxytetracycline, is used to treat bacterial septicaemia in farmed aquatic animals. However, treatment with these antibiotics, although effective, can have inherent risks. Not least the ability of pathogenic bacteria to develop resistance to the tetracyclines (Hameed et al, 2003). Furthermore, tetracyclines when exposed to light can form brown coloured toxic epi-anhydrotetracyclines which could impact negatively on survivability of the larvae. Evidence from fish aquaculture suggests that there is also a risk that tetracyclines could enter the waste water and select for resistant bacteria within this water (Björklund et al, 1990).

Therefore other means of disease management need to be investigated, including competitive exclusion, where the animal's own commensal microflora or probiotics are used to occupy the same ecological niche as the pathogenic bacteria to keep their number below the lethal dose. The addition of rapidly growing, non-harmful bacteria to the incubator system that are good producers of biofilms may also serve to limit the growth of *Aeromonas hydrophila*.

Therefore, the bacterial microflora that colonise the larvae and their hatchery environment may be very different to what is found in nature. It is known that the commensal microflora on animals can exert a protective function in several ways which include:

- Competition with potential pathogens for space and nutrients
- Stimulate the innate immune system
- Production of antimicrobial molecules

Redclaw larvae survive in the hatchery on the egg yolk without being fed up until their 3rd moult when they become Stage 3 Juveniles (S3J's or craylings). Thereafter they would either have to live off body reserves or need to start feeding. Possible sources of food in the hatchery where nutrients are not provided include the moult shells, cannibalism where either live, dead or moribund larvae are eaten, or ingestion of the bacterial biofilms found on the basket or free in the water. If the bacterial biofilm is a source of nutrition, then bacterial micro-colonies should be ingested and observed in the stomach. Furthermore, their numbers should be higher when the yolk has been fully utilised. The type and role of the microflora in redclaw health have not been extensively investigated, nor has the hatchery microbial dynamics over time been studied and how this may contribute to the health of redclaw larvae.

Several *Bacillus* species are used as probiotics in the redclaw hatchery. Although their role is considered to be similar to normal commensal microflora, their beneficial effects in a redclaw hatchery has not been fully determined.

Moreover, results from feeding trials done in Argentina on small scale incubated redclaw eggs (Stumpf *et al*., 2010) suggested craylings that have reached S3J (craylings, morphologically adults) must be fed within 4.28 days for 50 % to survive the first post juvenile moult. This has important implications for the egg incubation system as feeding within the egg incubators is problematic due to water quality issues. Craylings sometimes have to be kept for 1 and 2 weeks to accumulate enough numbers to meet commercial orders. Our own anecdotal evidence collected over the last few years does not support this thesis. However if the results of this study are reliable it has serious implications on survival of craylings older than 4.28 days when stocked from the incubators directly into ponds, hence these trials to determine the following:

- At what stage / age will hatched incubated eggs begin to feed?
- When is the critical time to start feeding craylings to ensure best survival?
- Do the larvae or craylings find something to eat within the incubator (bio-films / organic debris / each other)? If so, this could explain why our observations are different to those of Stumpf *et al.*, 2010
- Affect of control (no feed), commercial larval prawn diet and frozen live food on survival to first post juvenile moult

Following are some photos and explanations of the incubator and egg development for easier understanding of this research report.

Figure 1: 1 of 3 redclaw egg incubators at AquaVerde Redclaw

Figure 2: Eggs in baskets within the incubator

(A) Eyed egg just prior to hatch

(B) S1L, Stage 1 larvae hatching

(C) S2L, Stage 2 larvae

(D) S3J, Stage 3 Juvenile, morphologically adult

Figure 3: Images A, B, C and D show major stages of development

4. Need

This project addresses a part of the QFRAB research priorities for 2012 “Improve the survival & reliability of hatchling reared redclaw and develop mono-sex redclaw (male only offspring)”

It has also been identified by the Queensland Crayfish Farmers Assoc. (QCFA) and acknowledged by DAFF Queensland that for our redclaw industry to grow and develop beyond “extensive farming practices” we require a reliable hatchery for producing disease free seed stock all year round, which will lead to radical changes in farm husbandry which in turn will increase production and support the re-vitalisation of a stalled industry. It is estimated that changes brought about by use of a redclaw hatchery will improve production by 50% in the short term with the potential to double as industry becomes more experienced with the new farming methods.

In an attempt to fill the need for a redclaw hatchery, AquaVerde imported some technology (parts of a crayfish egg incubator) from Scandinavia and have modified it over the past five years to meet the biological and environmental requirements of redclaw. In partnership with the North QLD Farmers Association, James Cook University and DAFF Queensland we have achieved encouraging results inspiring us to move beyond the pilot stage and implement a “scalable” hatchery. However, despite our best efforts to resolve some problems in the hatchery we have made little progress in the last two years due to our lack of microbiological skills.

5. Objectives

1. Bacterial & fungal identification & management.
   Identify any bacteria or fungi that may be responsible for mortalities in hatched larvae and eggs. Identify the normal microflora in the hatchery, both in or on the animals as well as planktonic bacteria and those present in the biofilms over time.
2. Test commercial probiotics / develop in-house probiotics and best practice.
   To identify any possible candidates in the normal microflora that could be used as probiotics, should the commercial probiotics found not to be beneficial.
   In vitro properties of commercial probiotic will be investigated and the ability to produce bacteriocins as well as the ability to out-compete possible pathogens.
3. Determine critical time when hatched larvae need to start feeding.
4. Identify causes for unexplained mortalities at all life stages (egg, larvae, crayling).
5. Develop methods to identify viable eggs.

6. Methods

6.1 Sampling framework

Two periods of sample collections were done; the first on 10th and 11th March 2012 and on 6th August 2012: the second on 6th of November 2012, 12th December 2012 and 9th January 2013. The sampling on 10th and 11th March was a few weeks before the end of the hatchery cycle and the sampling on the 6th August was a few weeks into the beginning of a new hatchery cycle. All the samples, with the exception of a water sample originated from incubator 1 which was in operation at that time. A hatchery cycle refers to the time when an incubator is populated until it is depopulated and cleaned. The samples collected are shown in Table 1.

Table 1: Five sample collections of redclaw eggs and larvae on 10th and 11th March 2012, 6th August 2012, 6th November 2012, 12th December 2012 and 9th January 2013.

Date Number	10th and 11th March 2012	6th August 2012	6th November 2012	12th December 2012	9th January 2013
1	Live, embroyonated eggs from female after immersing in 10% formalin	Dead eggs	Dead eggs	Live eggs from a basket with dead eggs and larvae	Live eggs from healthy basket 1
2	Dead eggs	Recently dead eggs	Healthy and dead eggs	Live eggs from healthy basket	Live eggs from healthy basket 2
3	Healthy larvae	Live and dead eggs	Fail to hatch	Infertile Eggs	1st stage larvae
4	Sick larvae	Infertile eggs	1st stage larvae	Dead and live larvae	2nd stage larvae
5	Dead larvae	Live, embryonated eggs	2nd stage larvae	1st larvae	3rd stage larvae
6	Slime (floating biofilm) from the baskets	Dead eggs	Biofilm from the basket	2nd larvae	Biofilm from the basket
7	Adherent biofilm from the baskets	Healthy 1st stage larvae	Biofilm from the tube	Biofilm from the basket	Incubator water
8	Water after treatment prior to entering the incubators	2nd stage healthy larvae	Intake water	Incubator water	
9	Water in incubator 1	Slime	Incubator water	Intake water	
10	Water in incubator 2	Thin Biofilm			
11	Commercial probiotic	Thick Biofilm			
12		Water (Low speed flow)			
13		Water (In the system)			
14		Commercial probiotic			

6.2 Laboratory method

6.2.1 Culture of bacteria and fungi present in the eggs, larval stages and the incubator environment

6.2.1.1 Bacterial and fungal culture

Prior to culturing, all the Redclaw samples were washed three times in sterile, phosphate buffered saline (PBS) pH 7.4 and the biofilms and probiotic samples were streaked out directly. 100 ml of water was vacuum-pumped through a 0.45μm filter and the entrapped material on the filter was imprinted onto agar and streaked out.

Each egg and larvae sample was macerated with a sterile, cotton-tipped swab, the material collected with the swab and then streaked onto Columbia agar containing 5% sheep blood, MacConkey agar, Phenylethyl alcohol agar (PEA) and Sabouraud's dextrose agar (Oxoid Ltd., UK) containing chloramphenicol (Sigma-Altdritch, USA). A small portion of the sample was also placed in brain heart infusion broth (Difco laboratories, USA) to enrich those bacteria that were reluctant to grow directly on agar.

6.2.1.2 Bacterial subculture and identification

After 24 hours of incubation in air at 30°C, representative single colonies were streaked onto fresh blood agar and incubated under the same conditions as the original samples. Purified cultures were identified phenotypically and put in microbank tubes and placed in -80 ˚C freezer for storage. Phenotypic identification included the preliminary screening tests of Gram's stain, catalase, oxidase, spot indole and hanging drop motility tests. All Gram-negative bacteria were then further screened using API 20E (bioMerieux, France) which was incubated at 30°C, rather than 37°C. The primary aim was to use the test results which would be compared to identification tables that were compiled from various sources, the main being the most recent publications from The International Journal of Systematic and Evolutionary Microbiology dealing with *Aeromonas* and *Pseudomonas* species. The reason for this was that the database of the API 20E focuses on bacteria isolated from human tissues. In November the Biolog manual system (Biolog MicroStation™ System/MicroLog™ version 5.2.01, Hayward, USA) was purchased and thereafter this identification system with its much larger database on environmental bacterial was used to identify the isolated bacteria.

6.2.2 Histopathology

Ten dead larvae from 10th and 11th March 2012 were fixed in Davidson fixation which is recommended for most histological applications to reduces autolytic changes in crustacean samples (Lightner, 1996). After 48 hours in Davidson's fixation, crayfish larvae and eggs were collected and transferred to 70% ethanol, then dehydrated though a series of alcohols to xylene and then embedded in paraffin wax. Tissue sections (5 µm thickness) were cut and stained with haematoxylin and eosin (H&E) and Gram's stain. The sections were examined using a compound light microscope.

Bacteria in Gram's stained histological sections of redclaw larvae were counted under compound light microscopy using the 40X objective. When available, 10 larvae were counted per category i.e. 1st stage, 2nd stage, 3rd stage, healthy larvae, sick larvae and dead larvae per sampling. The hepatopancreas was examined using all possible fields without overlapping the fields. The bacterial number was ranked from 0 to 5 (see Table 2) and positive samples were calculated in percentages. The data was analysed using a Statistical Package for the Social Sciences (SPSS, PASW Statistics 20) program. The homogeneity of variance between groups showed that data were not normally distributed when the missing data were included. Therefore, the Kruskal Wallis test was used to analyse the data.

Table 2: Ranking key to give a semi-quantitative analysis of bacterial score in histological sections

Scores	Bacterial count
0	None
1	1 – 10 bacteria in total
2	10 – 50 bacteria in total
3	50 – 100 bacteria in total
4	100 – 500 bacteria in total
5	More than 500 bacteria in total

6.2.3 Partial DNA sequencing of cultured bacteria

6.2.3.1 DNA extraction

SV total RNA isolation system (Promega, Australia) was used for DNA extraction according to the manufacturer's instructions. Purified bacterial colonies were placed in 1.5 ml sterile tube. Lysis buffer was added and the solution was centrifuged at 13.2k x *g* through the 0.45µm filter. Then RNA/DNA free water was added and the bacterial DNA was directed through the filter into the new sterile tube.

6.2.3.2 PCR and sequencing

Primers were purchased that were able to partially amplify the 16S RNA gene common to all bacteria (Edwards *et al*., 1989) and the *rpo*D gene (Burton *et al*., 1981) and *aro*A gene (Huys *et al*., 2012) which are specific for *Aeromonas* (Table 3). Each 25 µL reaction consisted of template DNA, a primer set (reverse and forward primers) and PCR Master mix (GoTaq® Hot Start Polymerase, Promega, Australia) in the proportions recommended by the manufacturer. GeneTouch Thermal Cycler TC-E-96GA, Bioer, China, 2006 was used for PCR and the thermocycle parameters consisted of an initial denaturing step at 95°C for 4 min, followed by 35 cycles at 94°C for 30s and 60°C for 30s and 72°C for 1 min and finally 5 min at 72°C. PCR products were electrophoresed on 0.8% agarose gels. The PCR products were sent to Macrogen Inc. (Korea) for sequencing. The results from each bacterial DNA which consisted of three forward and three reverse sequences were analysed using Sequencher^{TM} software (Gene Codes Corporation, USA). The sequences were aligned using GeneDoc software version 2.6.002 developed by Nicholas, Karl B. and Nicholas, Hugh B. (1997) and then compared to available sequences using Basic Local Alignment Search Tool (BLAST), through the National Centre for Biotechnology Information (NCBI).

Table 3: Sets of primers for PCR

Number	Names of primers	Sequences
1	16S RNA Forward	CAGGCCTAACACATGCAACTC
2	16S RNA Reverse	GGGCGGWGTGTACAAGGC
3	*rpo*D Forward	AGTCAGGGTTCTGTDACAG
4	*rpo*D Reverse	GHGGCCARTTTTCHARRCGC
5	*aro*A Forward	TTTGGAACCCATTTCTCGTGTGGC
6	*aro*A Reverse	TCGAAGTAGTCCGGGAAGGTCTTGG

6.2.4 Inhibitory effect of commercial probiotics on *Chromobacterium* sp.

In the first sampling, there had been a high mortality of approximately 50% of S2L and *Chromobacterium* sp. was identified as the cause. Therefore, it was decided to check whether the commercial probiotic bacteria, *Bacillus pumilus* (A); *Bacillus licheniformis* (B); and *Bacillus subtilis* (C) were able to outgrow *Chromobacterium* sp. They were co-cultured on the nutrient agar that had been reconstituted with either sterile deionised water or tap water to compare the effect of mineral content. Dilutions of probiotics A, B, C, A+B, A+C, B+C and A+B+C from 1 to 10^{-6} dilution were made. *Chromobacterium* sp. was diluted to 10^4 cells per mL in sterile water and added to cooling molten nutrient agar (see table 9). The agar was poured into a Petri dish and allowed to solidify. Then, small wells were punched in the nutrient agar plate 10 µl of each probiotic dilution was added to a well. The plates were incubated overnight at 30˚C, observed and the diameter of a clear zone where bacterial growth was inhibited was measured around the probiotic dilution.

6.2.5 Inhibitor effect of probiotic candidates on *Aeromonas hydrophila*

Subsequent sampling revealed that the depopulation and cleaning procedure of the hatchery was able to remove the *Chromobacterium*. However, as had happened in samplings of the hatchery prior to the project, *A. hydrophila* had become dominant and appeared to be the cause of death in the larvae. Therefore, it was decided to repeat Experiment 6.2.4 using the isolated *A. hydrophila* instead of *Chromobacterium* and also test other probiotic candidates in a similar fashion.

The agar dilution method is only able to detect whether the probiotics are producing antimicrobial substances. Since this is not the only way in which bacteria interact competitively, it was decided to check whether probiotic candidates, *Acinetobacter genospecies* 6, *Acinetobacter grimontii* and *Chryseobacterium balustinum* could out-compete the pathogenic *Aeromonas hydrophila*. Three growth media were selected for the test, buffered peptone water (Oxoid Ltd., UK) reconstituted either with deionised water, sterilised tap water and sterilised farm water. One millilitre containing the selected probiotic at 10^4 cfu/ml and 1 ml of *A. hydrophila* at the same concentration were added to 8 ml of each growth medium and incubated in air at 30˚C. Bacterial counts were then performed daily on each dilution, using a 10-fold dilution series, with the Miles-Misra viable bacteria counting technique. It involved dispensing, in triplicate, 10 µl drops of each bacterial dilution on

Columbia blood agar and incubating overnight at 30˚C. After incubation, *A. hydrophila* and probiotic candidate bacteria were counted and recorded.

6.2.6 Feeding experiment

6.2.6.1 Determine if bacteria are utilised as a source of nutrition by larvae

This experiment was run on the different developmental stages of healthy redclaw larvae to determine if bacteria can be found in the intestinal tract and whether there were differences in the presence and quantity of bacteria along the intestinal tract in the different larval stages. Although several methods to determine this could have been used, due to funding constraints, it was decided that histological sections be made on larvae that had been fixed in Davidson's fixative and stained with haematoxylin and eosin (H&E) and Gram's stains. Furthermore, we tested when the yolk became completely digested and what other substances could be identified in the stomach contents.

Bacteria in Gram's stained histological sections of redclaw larvae were counted under compound light microscopy using the 40X objective (400X magnification). Ten larvae were counted per category i.e. early S2L, late S2L, early S3J and late S3J. The stomach, hepatopancreas and intestine were examined using all possible fields without overlapping the fields. The bacterial number was ranked from 0 to 5 (see table 2 in the bacterial culture experiment) and positive samples were calculated in percentages.

The data was analysed using a Statistical Package for the Social Sciences (SPSS, PASW Statistics 20) program. The homogeneity of variance between groups showed that the average bacterial percentage was normally distributed but not the score of bacteria. Therefore, the one way ANOVA was used to analyse the bacterial percentage data and the Kruskal Wallis test was used to analyse the score of bacterial colonisation data.

6.2.6.2 Determine critical time when hatched larvae need to start feeding

An experiment was run to determine the average survival of S3J's at 2, 7 and 14 days old with each age group being fed various feeds.

In this experiment, 135 S3J craylings were divided into 3 treatments; no feeding, commercial prawn larval diet, and frozen blood worms. Each treatment had 3 replicate groups with 15

animals in each group. Three trials were run using the same groupings, but with craylings where feeding commenced at either 2 days old, 7 days old or 14 days old. Each tank, of three replicates, had an area of 500 cm^2 and was stocked with 15 craylings, therefore a total of 45 craylings. The water temperature was maintained at 26°C, and the tanks were syphoned clean twice daily prior to feeding. Daily count of dead craylings, acts of cannibalism, missing and successful moults was made where possible.

Statistical Package for the Social Sciences (SPSS, PASW Statistics 20) program was used to analyse the survival data of the sum of the three replicates and compare survival in each of the three age groups (commencing feeding at 2 days of age, 7 days of age and 14 days of age). The statistical result showed that the data did not have a normal distribution. Therefore, Mann-Whitney U test was used to analyse the survival data between feeding and no feeding while Kruskal Wallis test were used to analyse the combined data.

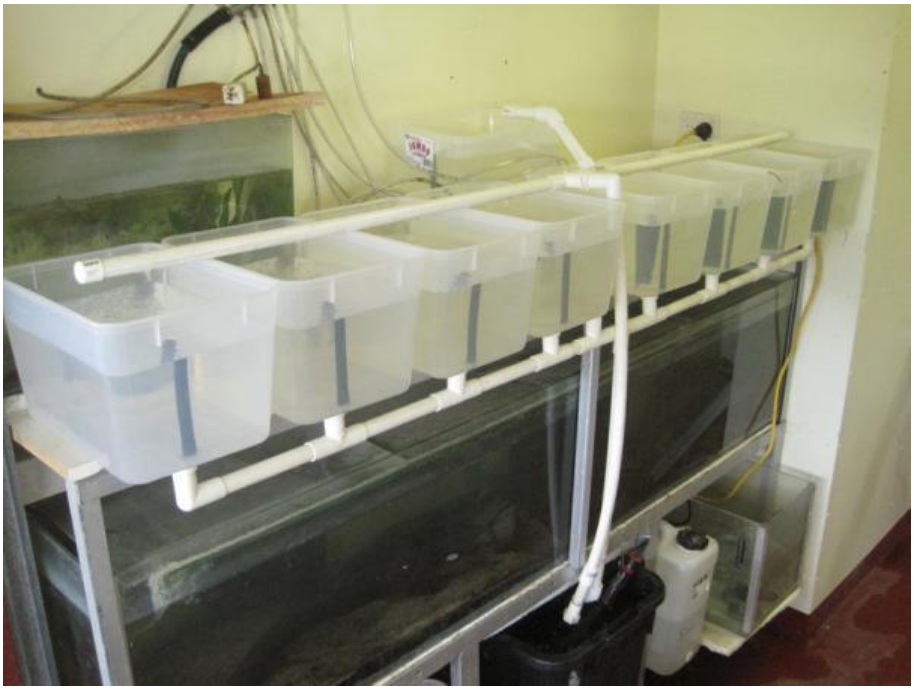

Figure 4: Set of 9 tanks for feeding trials

6.2.7 Identify viable eggs

During the course of this project, the eggs that failed to develop and hatch were collected to observe and identify the types of egg failures. Five main types of egg failure were catalogued, but time and financial constraints prevented us from investigating further at this time, however plans are under way to continue this work beyond the life of this project.

7. Results and discussion

7.1 Bacterial and fungal culture results

A summary of the bacterial cultures for each sampling and in each sample is provided in tables 4 and 5. The March 2012 sampling yielded, a non-haemolytic, Gram-negative, motile, catalase positive, oxidase- and indole-negative bacillus that predominated in the samples taken (refer to table 4). This bacterium was identified by 16S RNA sequencing to genus level as *Chromobacterium.* A non-haemolytic catalase-positive bacterium identified as *Pseudomonas putida* was cultured in lower numbers from only the dead eggs from the March samples and healthy larvae. *Aeromonas hydrophilia* was not cultured. *Penicillium* and *Gliocladium* were cultured from the hatchery water and biofilm. Ten bacterial colony types were cultured from 6th August 2012 samples, including a low number of *A. hydrophila*, however no fungus was found. Again the commercial probiotic bacteria were cultured from the water and biofilms, but not the eggs or larvae.

In November, the bacterial composition of the hatchery altered in that *Acinetobacter* species and *Pseudomonas aeruginosa* were isolated for the first time. Furthermore, *A. hydrophila* had become more dominant. In the December sampling a new *Acinetobacter* started to predominate, even being isolated from more samples than *A. hydrophila.* In this sampling, yeast was cultured from the larvae and biofilms, and three fungal colonies of *Fusarium* sp. were detected in the water samples. The January sampling yielded different bacteria, however, *Acinetobacter* genospecies 6 persisted and was the predominant bacterial species in that sampling. *A. hydrophilia* was not cultured.

Table 4: Bacteria isolated from 10th and 11th March 2012, 6th August 2012, 6th November 2012, 12th December 2012 and 9th January 2013 samples.

Date	Bacteria	Samples
10th and 11th March 2012	1. *Chromobacterium* sp. (A, P)	Dead eggs, Healthy, sick and dead larvae, slime, biofilm, water (intake and incubator)
	2. *Pseudomonas putida* group A (A)	Dead eggs and healthy larvae
6th August 2012	1. *Pseudomonas putida* group 1 (A)	Recently dead, live, dead and infertile eggs, healthy and S2L
	2. *Pseudomonas putida* group 2 (A)	Recently dead and live & dead egg mixture, live eggs and dead eggs
	3. *Pseudomonas putida* group 3 (A)	Infertile and dead eggs, healthy larvae
	4. *Pseudomonas putida* group 4 (A)	Live eggs and biofilm
	5. *Pseudomonas putida* group 5 (A)	Biofile from system
	6. *Pseudomonas aeruginosa* (A, P)	Biofilm from pipes
	7. *Aeromonas hydrophila* (A, P)	Dead eggs, healthy and S2L
	8. *Flavobacterium* sp. (A)	Dead eggs
	9. Non-fermentive Gram-negative bacillus 1 (A)	Live eggs
	10. Non-fermentive Gram-negative bacillus 2 (A)	Live eggs and biofilm
6th of November 2012	1. *Kingella denitrificans* (B)	Unviable, healthy & dead egg pool and fail to hatch eggs
	2. *Acinetobacter grimontii* (B, P)	Unviable, healthy & dead egg pool and fail to hatch eggs
	3. *Aeromonas hydrophila* (B, P)	Healthy & dead egg pool, 1st and 2nd stage larvae, biofilm and water (incubator)
	4. *Pseudomonas aeruginosa* (B, P)	Fail to hatch eggs, 1st and 2nd stage larvae, biofilm and water (intake and incubator)

12th December 2012	1. *Aeromonas hydrophila* (B, P)	Healthy, unhealthy and infertile eggs, 1st and 2nd stage larvae and biofilm
	2. *Acinetobacter* genospecies 6 (B)	Healthy, unhealthy and infertile eggs, dead and 2nd stage larvae, biofilm and water (intake)
	3. *Pseudomonas fuscovaginae* (B)	Water (intake)
9th January 2013	1. *Acinetobacter* genospecies 6 (B, P)	Healthy eggs from basket 1&2, 1st, 2nd and 3rd stages and biofilm
	2. *Herbaspirillum rubrisubalbicans* (B, P)	Healthy eggs from basket 1&2, 1st, 2nd and 3rd stages
	3. *Chryseobacterium balustinum* (B, P)	1st, 2nd and 3rd stage larvae
	4. *Pseudomonas aeruginosa* (B, P)	Water (incubator)
	5. *Elizabethkingia meningoseptica* (B)	Water (incubator)

Bacteria were identified by Api 20E test and identification tables (A) Biolog system test (B) PCR (P)

Table 5: A summary of isolated bacteria from 10th and 11th March 2012, 6th August 2012, 6th November 2012, 12th December 2012 and 9th January 2013 samples.

Date	10th &11th March 2012		6th August 2012										6th Nov 2012				12th December 2012			9th January 2013					Probiotic bacteria
Bacteria	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**J**	**F**	**O**	**F**	**P**	**O**	**Q**	**R**	**J**	**S**	
Dead eggs	X	X		X	X	X					X		X	X											X
Recently dead eggs			X	X																					
Live and dead eggs			X	X																					
Infertile eggs			X		X								X	X			X	X							
Eggs from unhealthy basket 1																	X	X							
Eggs from unhealthy basket 2																	X	X							
Live, embryonated eggs				X			X	X	X				X	X			X	X		X	X				X
Fail to hatch eggs													X	X	X										
Dead larvae	X															X	X	X							
Unhealthy larvae	X																								
Healthy larvae	X	X	X		X	X										X									X
1st stage larvae															X	X				X	X	X			
2nd stage larvae		X				X									X	X	X	X		X	X	X			
3rd stage larvae																				X	X	X			
Slime	X									X		X													X
Biofilm	X							X	X						X	X	X			X					X
Water (Intake)	X														X			X	X						
Water (Incubator)	X														X	X							X	X	X

A: *Chromobacterium sp.*, B: *Pseudomonas* species A, C: *Pseudomonas* species 1, D: *Pseudomonas* species 2, E: *Pseudomonas* species 3, F: *Aeromonas hydrophila*, G: Non-fermentive bacteria 1, H: Non-fermentive bacteria 2, I: *Pseudomonas* species 4, J: *Pseudomonas aeruginosa*, K: *Flavobacterium sp.*, L: *Pseudomonas* species 5, M: *Kingella denitrificans*, N: *Acinetobacter grimontii*, O: *Acinetobacter* genospecies 6, P: *Pseudomonas fuscovaginae*, Q: *Herbaspirillum rubrisubalbicans*, R: *Chryseobacterium balustinum*, S: *Elizabethkingia meningoseptica*

7.2 Histopathological results

No Gram negative or Gram positive bacteria were detected the hepatopancreas of healthy redclaw larvae after staining with Gram's stain (figure 5). One out of ten dead larvae from 10th and 11th March 2012 and 6th August 2012 samples had Gram-negative rod-shaped bacteria associated with inflammatory cells in the hepatopancreas after the sections were stained with Gram's stain (figures 6 and 7). Some larvae had many bacterial colonies within and outside of the hepatopancreatic tissues. No bacteria or fungi were apparent in other organs and tissues of infected larvae.

First and second stage larvae from 6th November 2012 and 12th December 2012 samples had Gram-negative rod-shaped bacteria with inflammatory cells in the hepatopancreas (figure 8). Larvae had many bacterial colonies within and outside of the hepatopancreatic tissues (figure 9 and 10). However, no bacteria or fungi were apparent in other organs and tissues of healthy redclaw crayfish from 9th January 2013 samples.

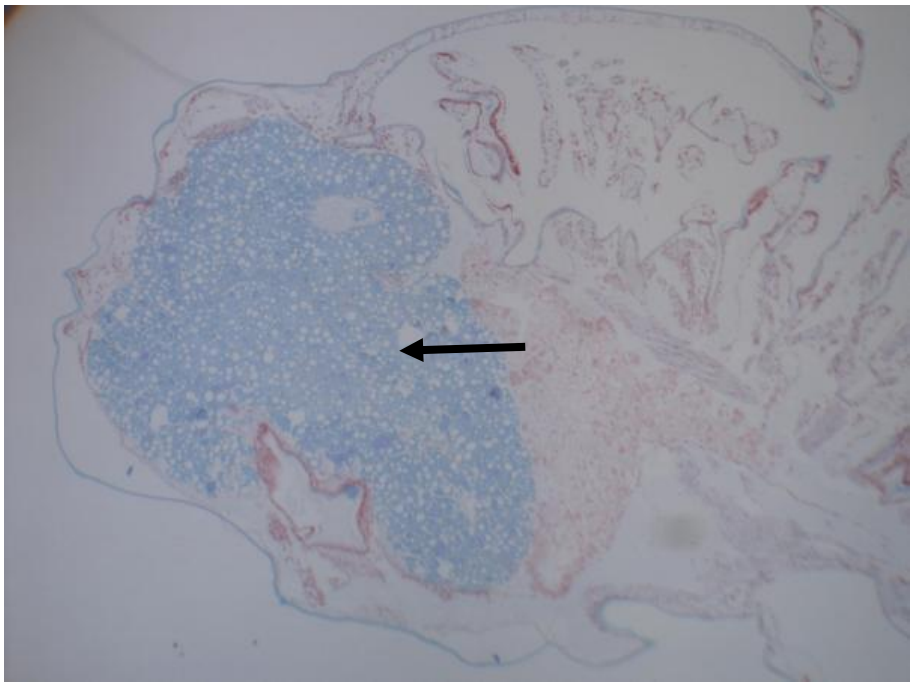

Figure 5: The hepatopancreas as indicated by the arrow of redclaw larvae were stained with Gram's stain which is used to detect and identify positive and negative bacteria. However, no positive or negative bacteria were detected in healthy redclaw larvae.

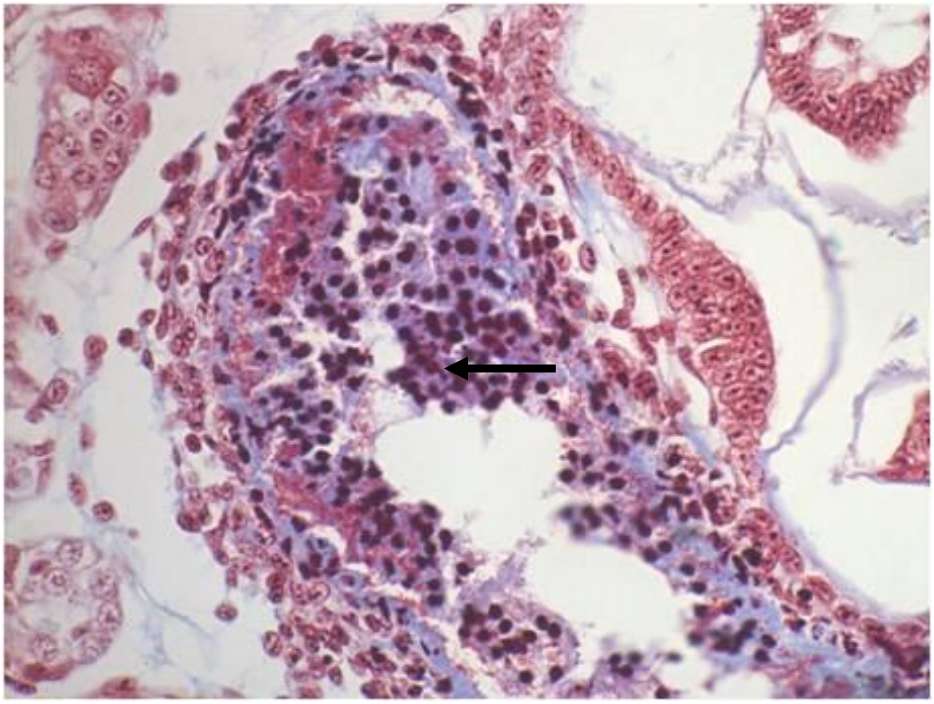

Figure 6: Bacteria with inflammatory cells (arrow) in the hepatopancreas of dead larvae (*Cherax quadricarinatus*) stained with Gram's stain.

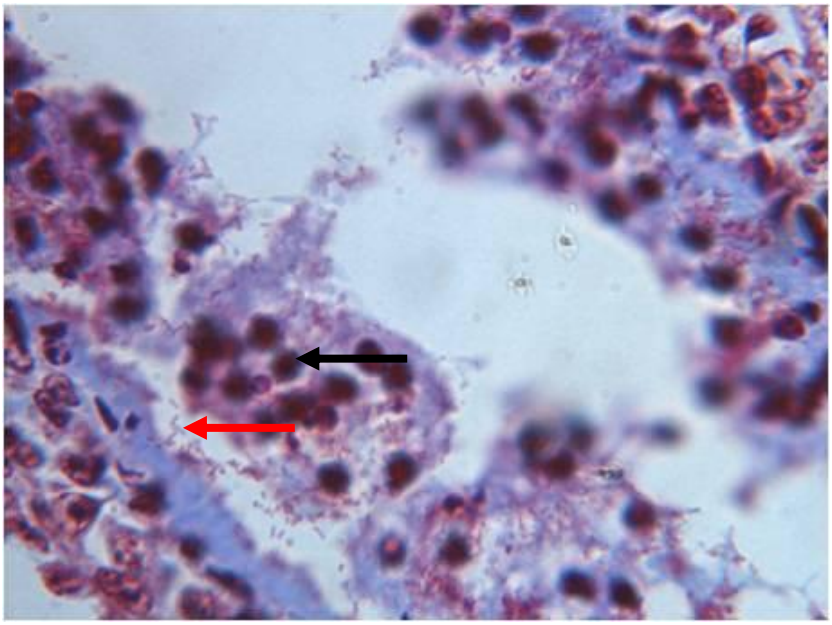

Figure 7: Gram-negative rod-shaped bacteria (red arrow) with inflammatory cells (black arrows) were found in the hepatopancreas of dead larvae of *Cherax quadricarinatus* stained with Gram's stain.

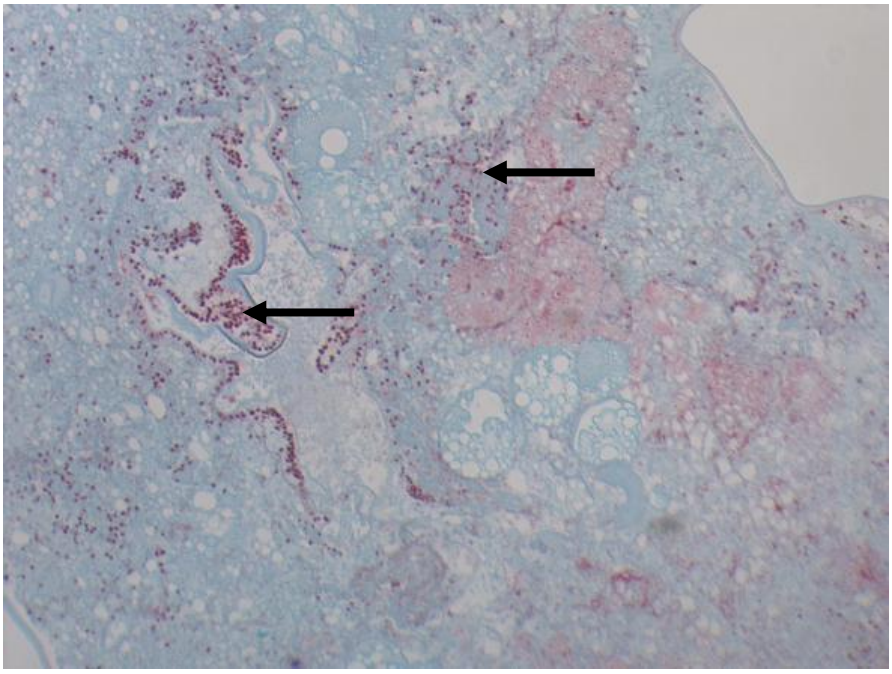

Figure 8: Gram-negative rod-shaped bacteria with inflammatory cells (arrows) in the hepatopancreas of larvae stained with Gram's stain

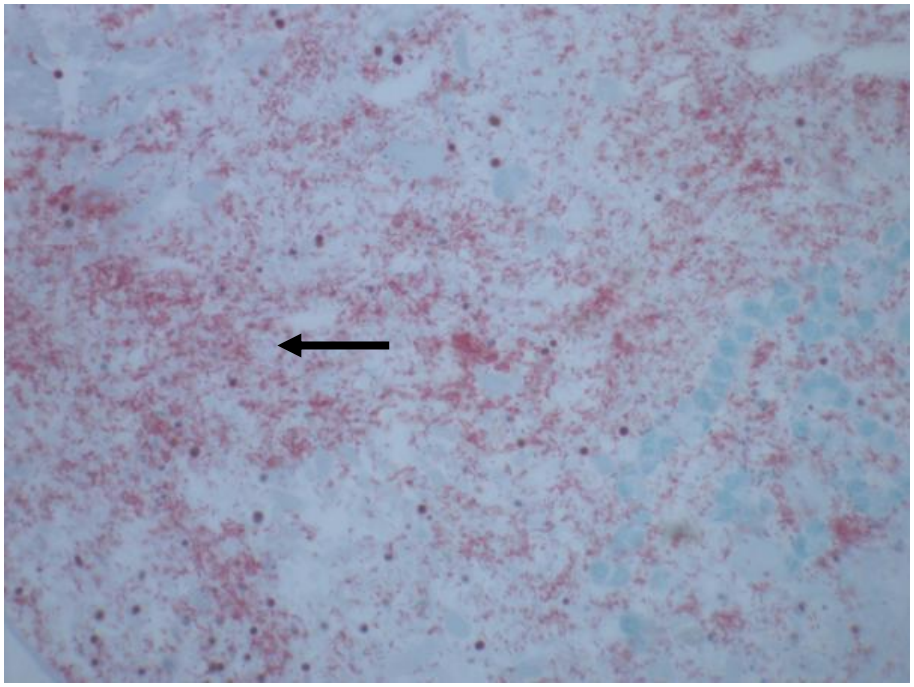

Figure 9: Numerous Gram-negative rod-shaped bacteria (arrow) in the hepatopancreas of larvae stained with Gram's stain

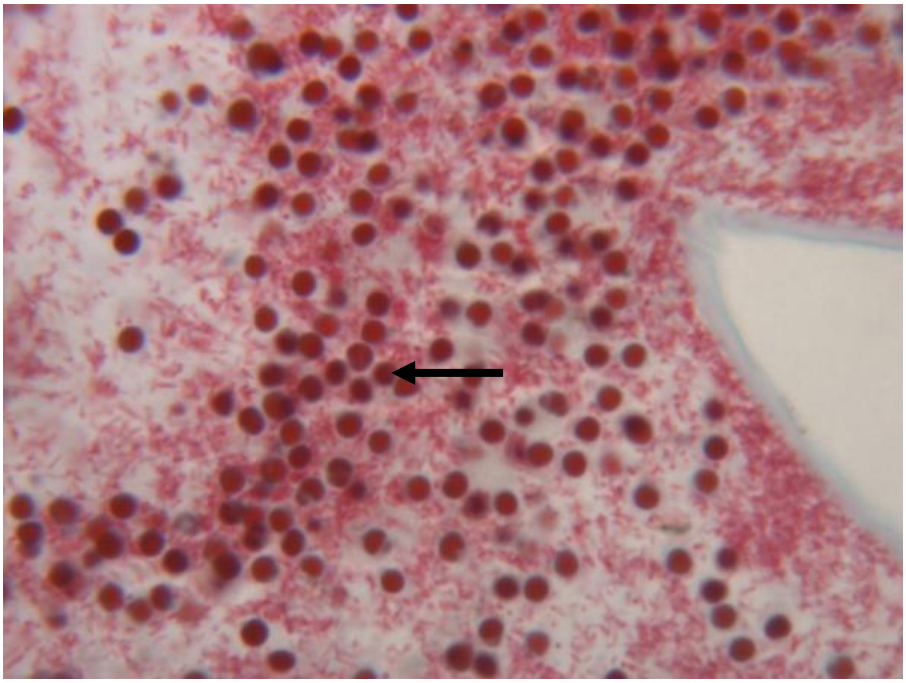

Figure 10: Gram negative rod-shaped bacteria with inflammatory cells (arrow) in the hepatopancreas of larvae stained with Gram's stain

Average scores of the bacterial numbers are presented in table 2. Not only did dead (1.7 out of 5) and then sick larvae (0.8 out of 5) have higher numbers of bacteria than the healthy larvae of the same age (0.1 out of 5) (table 6), but more dead and sick larvae were colonised with bacteria than the healthy larvae. (table 7). However, when the results from all stages of healthy larvae are combined and compared to that of the sick and dead larvae, it was found that there was no statistical difference in bacterial score ($P = 0.354$) or bacterial colonisation percentage ($P = 0.201$) of the hepatopancreas. However, what was determined was that all the dead and sick larvae had evidence of inflammatory cell infiltration of the hepatopancreas (referring to figures 6, 7, 8 and 10), which was absent in the healthy larvae of all stages.

Table 6: Summary of average observable bacterial scores from all redclaw crayfish larval samples

Date Samples	10th and 11th March 2012	6th August 2012	6th November 2012	12th December 2012	9th January 2013
S1L	-	0.5	0.7	1.9	1.2
S2L	-	0.2	1.6	2.7	1
S3J	-	-	-	-	0.6
Healthy larvae	0.1	-	-	-	-
Sick Larvae	0.8	-	-	1.9	-
Dead Larvae	1.7	-	-	-	-

Table 7: Percentages of redclaw larvae that had observable bacteria in the hepatopancreas

Date Samples	10th and 11th March 2012	6th August 2012	6th November 2012	12th December 2012	9th January 2013
S1L	-	40	40	90	50
S2L	-	20	80	90	60
S3J	-	-	-	-	40
Healthy larvae	10	-	-	-	-
Sick Larvae	50	-	-	100	-
Dead Larvae	70	-	-	-	-

What is clear from this small data set of results is that there appears to be no statistical difference between the bacterial numbers in hepatopancreas of healthy compared to sick or dead larvae. The constant changing conditions of the hatchery tanks, the fact that the hatchery is not active throughout the year, and budgetary constraints led to the number of samples being smaller than what was originally planned for. Whether the picture would be different with a larger sample size is uncertain. However, when comparing the level of inflammation between the healthy and sick or dead larvae, it is clear that the sick animals where producing an inflammatory response to the bacteria, whereas the healthy larvae were tolerating the bacteria.

7.3 PCR and sequencing

DNA of cultured bacteria from 10th and 11th March 2012, 6th August 2012, 6th November 2012, 12th December 2012 and 9th January 2013 samples was extracted, amplified using the PCR method and the amplicons visualised by gel electrophoresis as bands of approximately 1000 base pairs (figure 11). The amplicons were sent for purification and sequencing. Sequencing result determined the bacterium from 10th and 11th March 2012 samples was *Chromobacterium* sp using 16S and *rpo*D primers. Also, *aro*A primer was used to confirm *Aeromonas* sp from 6th August 2012 samples. The identification results from API20E test, Biolog system and sequencing were compared and shown in table 8.

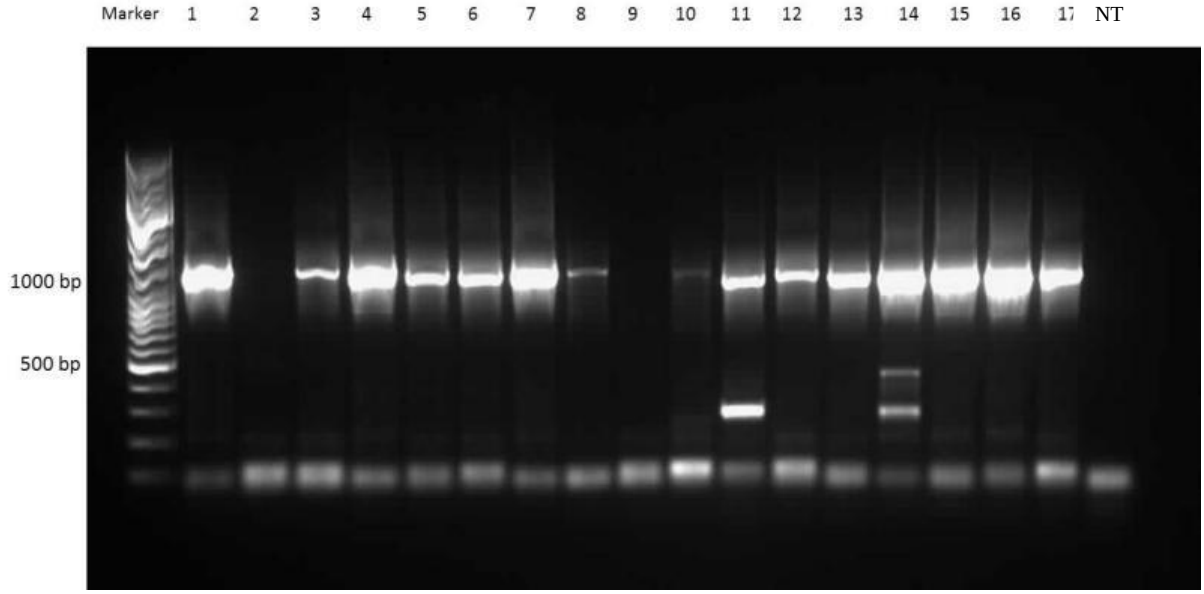

Figure 11: DNA products of bacterial samples from PCR were run on 0.8% agar gel. Samples 1 to 8 were amplified using 16S RNA primers, while *aro*A and *rpo*D primers were used for samples 9 to 13 and 14 to 17, respectively. NTC: not template control.

Table 8: Sequencing results of bacteria isolated from 10th and 11th March 2012, 6th August 2012, 6th November 2012, 12th December 2012 and 9th January 2013 samples.

Date	Api 20E or Biolog system	PCR & Sequencing
10th and 11th March 2012	1. *Chromobacterium* sp.	*Chromobacterium* sp.
	2. *Pseudomonas putida* group A	*Pseudomonas*
6th August 2012	1. *Pseudomonas putida* group 1	*Pseudomonas*
	2. *Pseudomonas putida* group 2	*Pseudomonas*
	3. *Pseudomonas putida* group 3	*Pseudomonas*
	4. *Pseudomonas putida* group 4	*Pseudomonas*
	5. *Pseudomonas putida* group 5	*Pseudomonas*
	6. *Pseudomonas aeruginosa*	*Pseudomonas*
	7. *Aeromonas hydrophila*	*Aeromonas hydrophila*
	8. *Flavobacterium* sp.	NA
	9. Non-fermentive bacteria 1	NA
	10. Non-fermentive bacteria 2	NA
6th of November 2012	1. *Kingella denitrificans*	NA
	2. *Acinetobacter grimontii*	*Acinetobacter*
	3. *Aeromonas hydrophila*	*Aeromonas hydrophila*
	4. *Pseudomonas aeruginosa*	*Pseudomonas*
12th December 2012	1. *Aeromonas hydrophila*	*Aeromonas hydrophila*
	2. *Acinetobacter* sp.	*Acinetobacter*
	3. *Pseudomonas fuscovaginae*	*Pseudomonas* sp
9th January 2013	1. *Acinetobacter sp.*	*Acinetobacter*
	2. *Herbaspirillum rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*
	3. *Chryseobacterium balustinum*	*Chryseobacterium balustinum*
	4. *Pseudomonas aeruginosa*	*Pseudomonas* sp
	5. *Elizabethkingia meningoseptica*	NA

7.4 Probiotic test

7.4.1 Commercial Probiotic tests

The bacterial lawn of *Chromobacterium* grew normally in the presence of all the commercial probiotic combinations, namely, A, B, C, A+B, A+C, B+C and A+B+C, irrespective of the dilution of probiotic used (see table 9). None could control or stop growth of *Chromobacterium*. The same result was obtained for *A. hydrophila*.

The commercial probiotics consisted of three different *Bacillus* species, which are used as probiotics in fish and prawn aquaculture. However, in this trial, *Bacillus* species were not able to out-compete either *Chromobacterium* or *Aeromonas hydrophila*. This was evidenced by the fact that only after the tanks had been cleaned and supplied with fresh clean water would *Bacillus* species be the dominant species of the biofilms. Once the tanks had been in operation for a while and *Aeromonas hydrophila* had been introduced, it replaced the *Bacillus* species and became the predominant bacterium both in the animals and in the biofilm. Further evidence was that the agar diffusion trial showed that none of the *Bacillus* species produced diffusible substances that could inhibit *Chromobacterium* or *Aeromonas hydrophila.* Furthermore, when the *Bacillus* species and *Aeromonas hydrophila* were co-cultured, the *Aeromonas hydrophila* grew faster than the *Bacillus* species and within 48 hours of co-culture was isolated in pure culture. A similar study was done using some of the pseudomonads that had been isolated from the biofilms when *Aeromonas hydrophila* was present in low numbers. They too failed to out-compete the voracious *Aeromonas hydrophila*.

7.4.2 Probiotic candidate test on *Aeromonas hydrophila*

The growth of *A. hydrophila* was not inhibited by any of the probiotic candidate bacteria, *Acinetobacter* genospecies 6, *Acinetobacter grimontii*, *Chryseobacterium balustinum* when they were co-cultured on reconstituted nutrient agar with sterile deionised water, tap water or farm water. When co-cultured in buffered peptone water, the probiotic candidates could still be recovered after 24 hours of incubation 28°C. However, by 48 hours after incubation, only *A. hydrophila* could be recovered, inferring that the probiotic candidates were not able to out compete the *A.hydrophila* under ideal growth conditions. After 4 days of incubation it was difficult to count the number of *A. hydrophila* at dilution 10^{-15} (table 9).

Table 9: Results of co-culturing of the probiotic candidate bacteria (*Acinetobacter* genospecies 6 (A), *Acinetobacter grimontii* (B), *Chryseobacterium balustinum* (C)) with *Aeromonas hydrophila*.

	Day 1		**Day 2**		**Day 3**	
	A. hydrophila	**Probiotic candidates**	***A. hydrophila***	**Probiotic candidates**	***A. hydrophila***	**Probiotic candidates**
Buffered peptone dd water *A. hydrophila* / A	3×10^{11}	0 (A)	4×10^{12}	0 (A)	7×10^{13}	0 (A)
Buffered peptone dd water *A. hydrophila* / B	9.1×10^{12}	1×10^{11} (B)	9.5×10^{14}	0 (B)	9.1×10^{14}	0 (B)
Buffered peptone dd water *A. hydrophila* / C	9.11×10^{12}	1×10^{11} (C)	9.1×10^{13}	0 (C)	1.71×10^{15}	0 (C)
Buffered peptone Tap water *A. hydrophila* / A	9.11×10^{12}	1×10^{11} (A)	1.12×10^{13}	0 (A)	2.18×10^{15}	0 (A)
Buffered peptone Tap water *A. hydrophila* / B	2.6×10^{12}	0 (B)	2.8×10^{13}	0 (B)	1.82×10^{15}	0 (B)
Buffered peptone Tap water *A. hydrophila* / C	2.1×10^{12}	0 (C)	6.8×10^{13}	0 (C)	1.11×10^{15}	0 (C)
Buffered peptone Farm water *A. hydrophila* / A	2.11×10^{13}	1×10^{12} (A)	1.38×10^{13}	0 (A)	1.33×10^{15}	0 (A)
Buffered peptone Farm water *A. hydrophila* / B	2.1×10^{13}	1×10^{11} (B)	2.22×10^{13}	0 (B)	1.19×10^{15}	0 (B)
Buffered peptone Farm water *A. hydrophila* / C	1.41×10^{13}	1×10^{11} (C)	2.45×10^{13}	0 (C)	1.48×10^{15}	0 (C)

7.5 Determine if bacteria are utilised as a source of nutrition by larvae

The average bacterial scores of the stomach, hepatopancreas and intestine are shown in table 10. The bacterial scores in the stomach (P = 0.004) and intestine (P = 0.005) were found to be significantly different between the groups of larvae. No significant differences were noted between the bacterial scores in the hepatopancreas (P = 0.231) between the different groups.

Table 10: Average bacterial scores in the stomach, hepatopancreas and intestine of redclaw

	Total positive	**Stomach**	**Hepatopancreas**	**Intestines**
Early S2L	0.87	0.7	1.8	0.1
Late S2L	0.73	1.1	0.0	1.1
Early S3J	0.51	0.1	0.6	0.8
Late S3J	0.29	0.4	0.4	0.1

The percentage bacterial colonisation of larvae is shown in table 11 and figure 12. The percentage colonisation of each organ was not significantly different between the different groups of larvae (P = 0.359). However, the S3J's had fewer numbers of bacteria than the S2L's (see figure 12). The results also demonstrated that the hepatopancreas of more early S2L's had been colonised than other larval groups. Interestingly, there were no bacteria in the intestines of early S2L's and the hepatopancreas of late S2L's. This result was also reflected in the score.

Table 11: Percentages of redclaw larvae colonised with bacteria in their intestinal tract

	Total positive	**Stomach**	**Hepatopancreas**	**Intestines**
Early S2L	90	40	70	10.0
Late S2L	72.7	66.7	0.0	57.1
Larly S3J	60.0	16.7	42.9	50.0
Late S3J	29.4	29.4	35.3	20.0

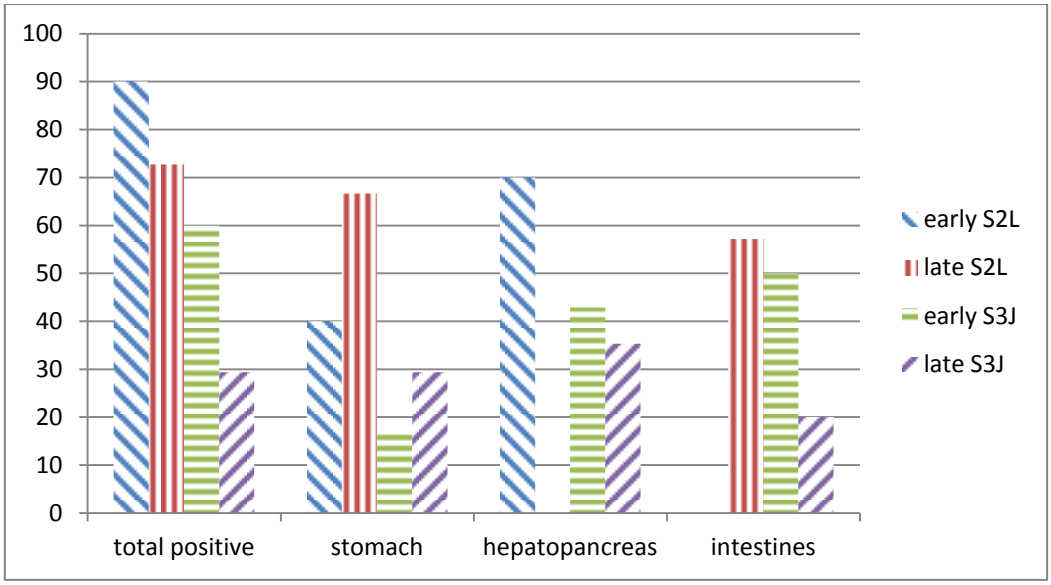

Figure 12: Graph showing percentages of redclaw larvae colonised with bacteria in their intestinal tract

In addition, it was also observed that the yolk was present in all early and late S2L, in 6 out of 10 early S3J's and none of the late S3J's (figure 13 shows H&E staining of the yolk of an early S2L). Pieces of carapace were observed to be in the stomach of 8 of the 10 Late S3J's (figure 14 shows PAS stain of the stomach contents of a late S3J).

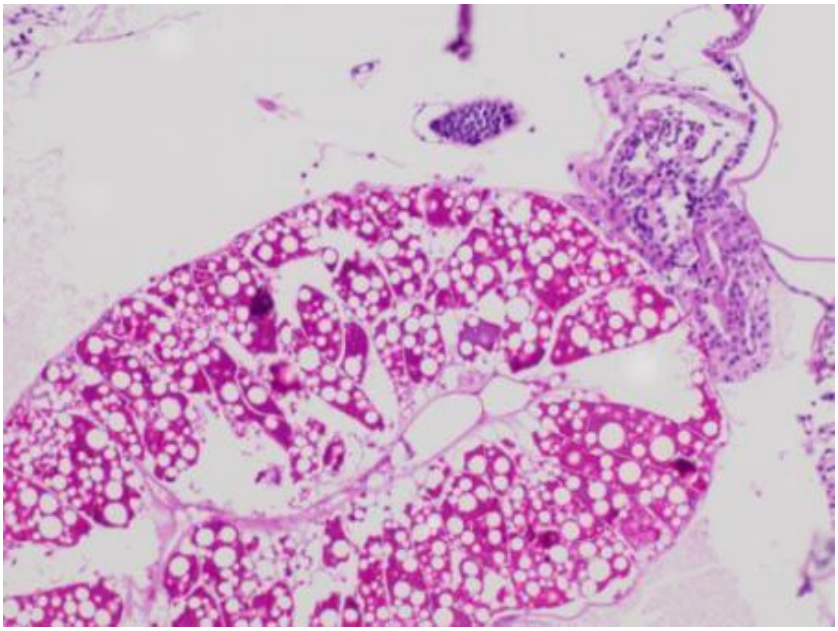

Figure 13: Yolk of an early S2L stained with H&E stain.

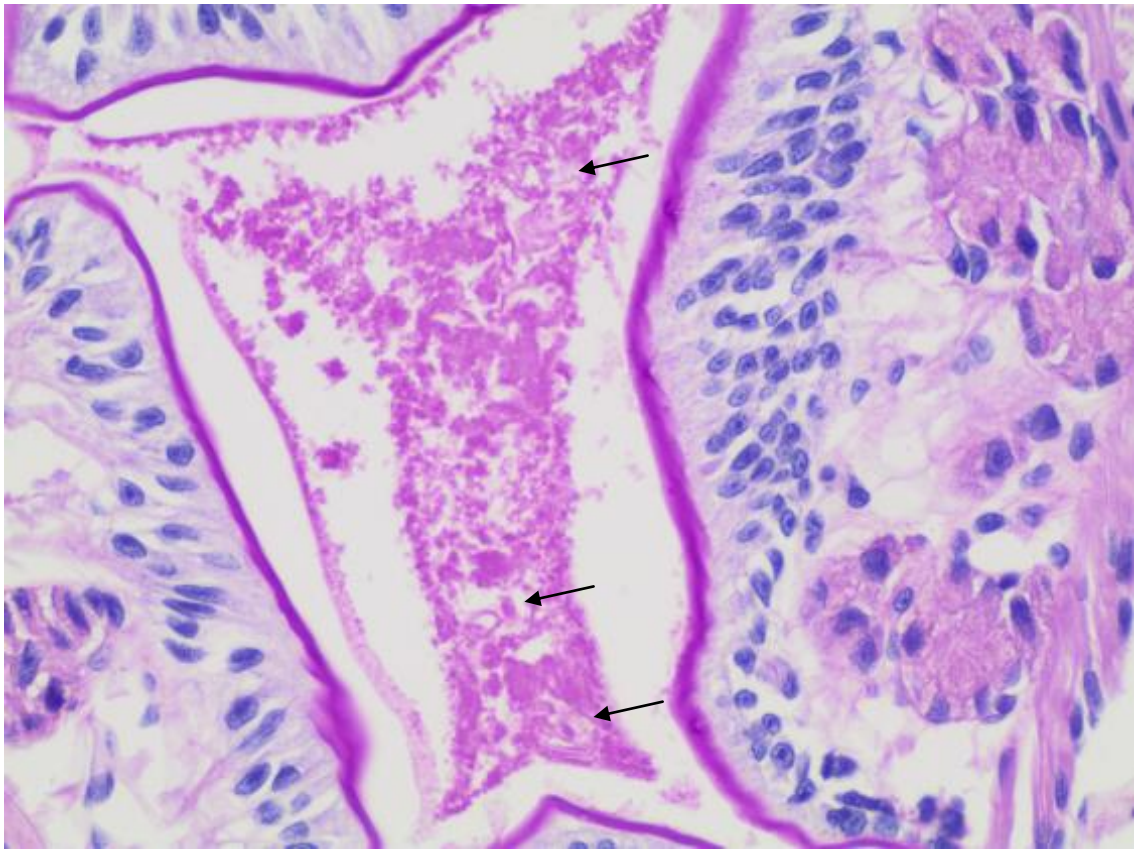

Figure 14: A histological section of the stomach of late S3J stained with Periodic Acid Schiff (PAS) stain to detect carbohydrates. It shows the presence of parts of exoskeleton (as indicated by the arrows) in the stomach.

This is further evidenced by the fact that the larvae and craylings ingested exoskeleton. We could not determine whether the carapace originated from moult shells or cannibalised dead or live siblings. Close and continuous observation of larval behaviour during this stage would most probably be the best way to answer this question.

Regarding the presence of bacteria in the intestinal tract, we found that due to the small numbers of samples statistical significance could not be reached. However, there was a clear tendency for larger numbers of bacteria to be found in the stomach of S2L's. This may represent ingestion of the bacteria since large microcolonies in a pink granular matrix were observed in the stomach of some of the larvae (refer to figure 15 of the stomach a Gram's stain of a late S3J). What was surprising was that the numbers of bacteria in the stomach decreased in S3J's compared to S2L's. It is uncertain whether this decrease is correlated with the production of acid by the granular stomach.

In this experiment, 1+ bacteria were present in the intestine of only one of the early S2L, indicating that bacteria had not yet passed into and colonised the small intestine. The late S2L had very high numbers, again reflecting the higher intake of bacteria at this time. Of interest was the high numbers of bacteria in the hepatopancreas of early S2L. This could be due to the fact that the local innate immunity was immature or that enzyme producing cells of the hepatopancreas were less vacuolated (see figure 16) than later stages indicating a lower digestive capacity. It should also be noted that there was no histological evidence of inflammation in the hepatopancreas of these larvae. What we cannot explain is the lack of bacteria in the hepatopancreas of the late S2L, which was in spite of the fact that high numbers of bacteria were present in the stomach and intestines.

Therefore it appears that S2L are able to obtain nutrition from both the egg yolk and bacteria in biofilms. The S3J's with the depletion of egg yolk and relying less on bacteria have turned to other sources of nutrition; for example, the exoskeleton. From this work, it is believed that the S3J's would benefit from being fed as soon as possible.

The hatchery has had batches of larvae during the study period, i.e. March 2012, where high mortalities were observed in predominantly the late S2L. It appears that it is the early S2L where the hepatopancreas is exposed to higher numbers of bacteria. If it is found that pathogenic bacteria are part of that microflora, as isolated in the bacterial culture experiment, it could be theorised that inflammation and septicaemia could result.

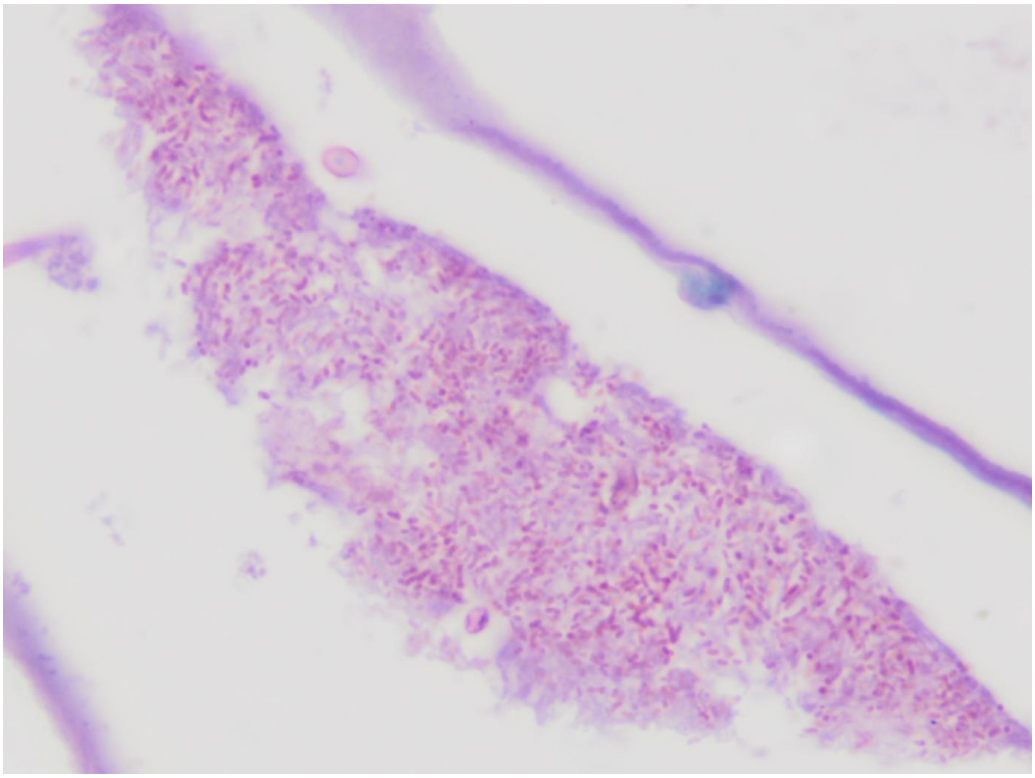

Figure 15: A histological section of the stomach of early S2L stained with Gram's stain. It shows the presence of large numbers of Gram-negative rods present in a pink matrix, most likely a biofilm.

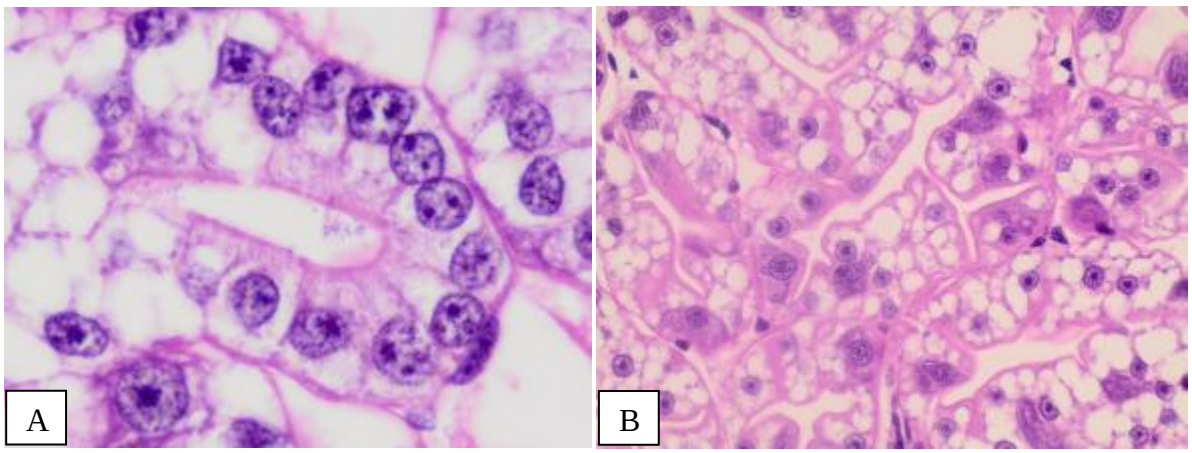

Figure 16: Histological sections of the hepatopancreas of A) early S2L and B) late S3J, showing greater vacuolation of the hepatopancreatic cells of the late S3J.

7.6 Determine critical time when hatched larvae need to start feeding

Although the control tanks were kept as clean as possible the control craylings showed just visible signs of having eaten something (the old farmer adage that it's impossible to starve a crayfish seems to apply) (figure 17).

(A) Fed S3J (crayling) (B) Crayling, fresh from incubator (not yet fed) (C) Stage 2 larvae feeding on dead sibling

Figure 17: Picture (A) shows a fed crayling compared to (B) a crayling fresh from the incubator. (C) Shows a stage 2 larvae feeding on dead sibling

Figures 18, 19 and 20 show the survival of S3J craylings in the feeding experiment using no feed, prawn feed and blood worm. For all 3 trials the fed animals, irrespective of the type of food fed (prawn dust or bloodworms), had a higher survival rate than the control animals that were not fed ($P < 0.05$). There was no statistical differences between the two different feed types (prawn dust and bloodworms) when feeding had started at 2 and 7 days of age ($P = 0.146$ for 2 day S3J's and $P = 0.582$ for 7 day old S3J's). These results are clearly illustrated in figure 18 and 19. However, there was a difference in the feeds when feeding had been initiated on 14 day old S3J's where the prawn dust resulted in a significantly better survival rate ($P = 0.007$) than the bloodworm. This is illustrated in figure 20.

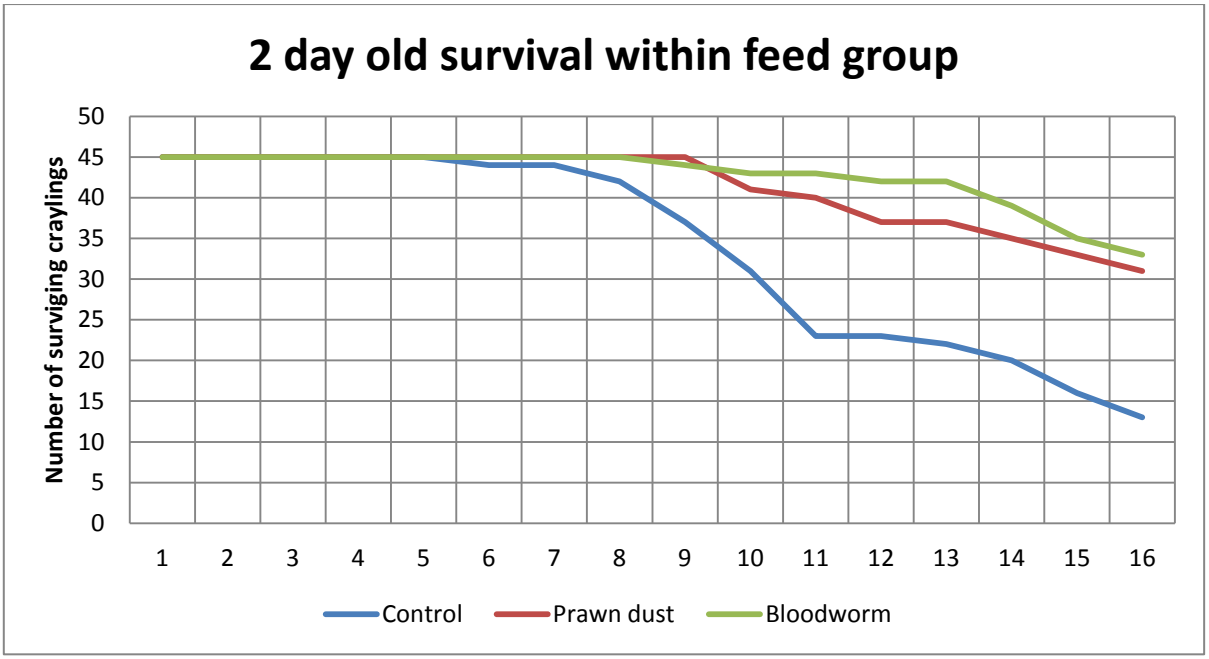

Figure 18: Graph shows the survival of S3J's that were fed from 2 days of age

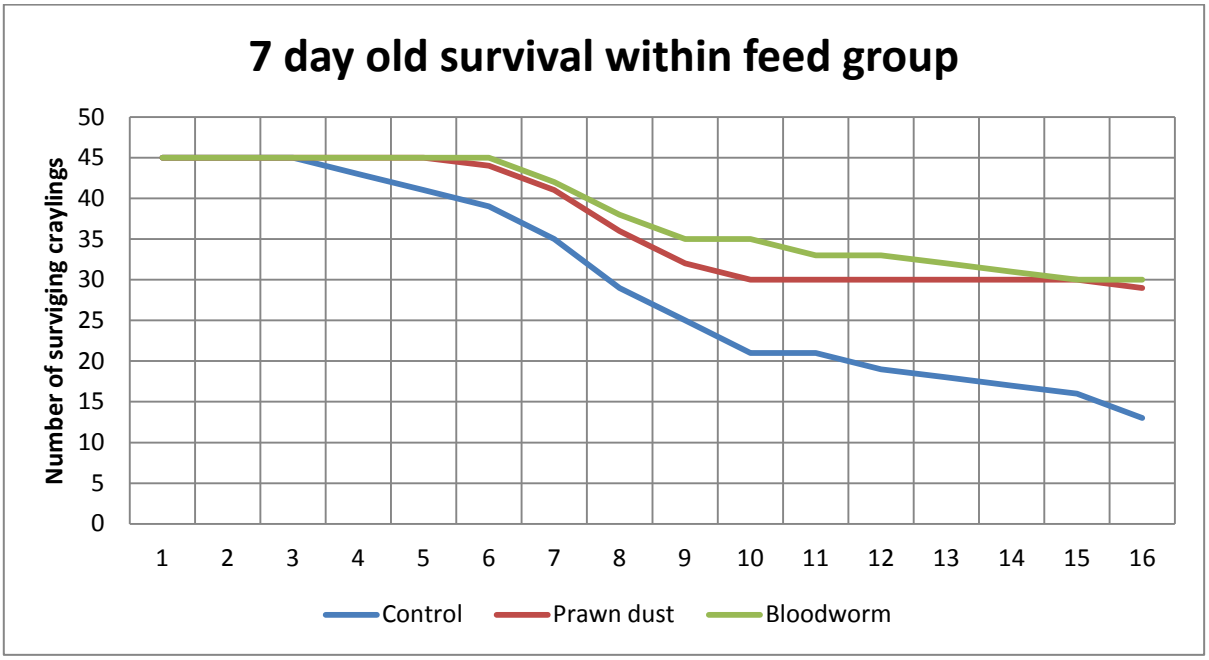

Figure 19: Graph shows the survival of S3J's that were fed from 7 days of age

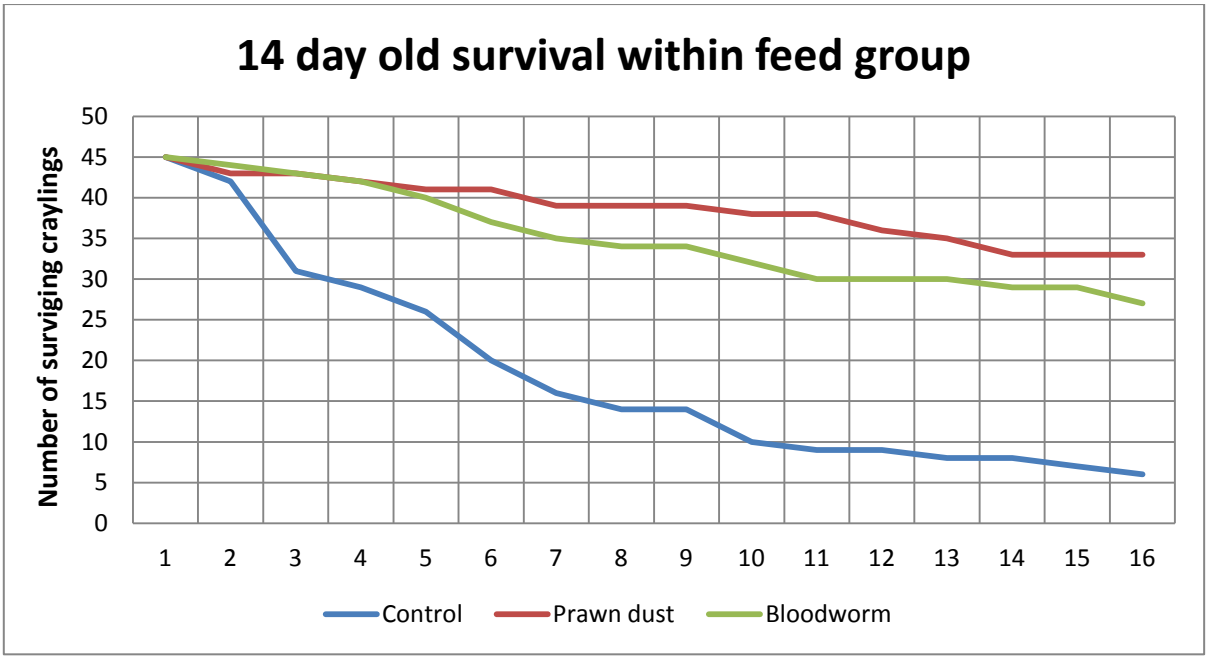

Figure 20: Graph shows the survival of S3J's that were fed from 14 days of age

Irrespective of the age at which feeding had started, the survival rate of the S3J craylings was much higher when fed than when they were not fed. However, it does appear that the survival rates of the S3J's were the same at the end of the feeding trial, irrespective of the age they were when feeding had started. This indicates that S3J craylings should be fed, but it may not be necessary to feed them before they are 12 days of age.

The types of feed when fed to 2 day old and 7 day old S3J's at the start of the feeding trials did not have any significant effect on their survival, however, the prawn dust performed significantly better, resulting in a 13% (6 out of 45 craylings) better survival, than blood worms when 14 day old S3J's had been fed for the first time.

7.7 Identify viable eggs

Through the course of this project five types of eggs failed to develop to hatch; four of them are pictured below in figure 21. So far they include bacterial infection, *Epistylus*, fertilised but fail to develop (reasons still unknown), *Saprolgenai/Fusarium* and unfertilised (not pictured). Time and financial constraints prevented us from investigating further at this time; however plans are under way to continue this work beyond the life of this project (see chapter 9: Further development)

Healthy late stage egg | Internal *Aeromonas* sp infection | *Epistylus* sp | Fertilised but failed to develop | *Saprolegnia / Fusarium*

Figure 21: Healthy egg and 4 types of failed eggs

8. Benefits and adoptions

Production in the hatchery has already significantly improved due to a greater understanding of the microbiological implications researched in this project. This is demonstrated in better management of the micro flora / fauna within the egg incubators, which leads to more consistency in survival and reliability.

The extra hatchery production has been immediately taken up by redclaw growers with increasing demand for next season. These farmers have already begun to adapt their farming practices around the use of "batch in batch out" crayfish farming.

Farm husbandry

- Increased yields and extending crayling production season to 9 months
- Increased profitability for growers by freeing them up from using brood stock or juvenile ponds
- Improved efficiency by simplifying production, reducing duplication and work load on the farmer
- For the first time farmers are able to stock exact quantities of same age craylings and so will be better able to predict growth rates and bio-mass, to better manage feeding rates / water exchange and predict production
- Facilitate the move from an extensive to a more intensive aquaculture industry
- Earlier stocking of craylings in spring to increase summer grow-out period which is particularly useful for southern growers.

Disease mitigation

- Breaking vertical disease transmission, improving health of crayfish
- Allow the movement of craylings from farm to farm without spreading disease
- Increase in survival
- More uniform growth rates

Re-vitalising a stalled industry

- Through our growing knowledge and expertise we have been providing more guidance and consultation to growers concerning the use of craylings.
- New entrants to the industry can save the time it would take to grow their own brood stock and can have an immediate start up with hatchery produced craylings.
- Increasing production (more profitable for producers)
- Farmers report reduced work load
- This technology has already allowed the North QLD Crayfish Farmers Association (NQCFA) to implement a selective breeding project that would have been far less efficient (with less control over family lineage) if traditional crayling production methods were used. As we have obtained a high level of confidence AquaVerde has now taken over the selective breeding project with the incubation process being the pivotal part of the process.

9. Further development

Although this research project has not covered all intended aims due to financial and time restrictions; it has provided a solid base in which to continue work on the stated objectives with the aim of solving hatchery technical problems.

Because of the changing nature of micro-organism ecology within artificial systems it is essential that hatchery operators continue to develop techniques to head off as many disasters as possible before they occur.

We have began the process of utilising Master's and PhD students to build on the results of this project by extending the research in the laboratory and performing more *in vivo* experiments in the hatchery. In particular in the areas of bacteria management and improving egg survival by:

- Continuing the search for probiotic species for competitive exclusion of pathogenic bacteria and ongoing experimental developments of techniques that help manage / reduce pathogenic organisms.
- Systematically devise methods to identify viable eggs with the aim of developing techniques to minimise the loss.
  Although five main causes of egg failure were identified during this project (bacterial infection, epistylis, fertilised but failing to complete development [reason still unknown] saprolegnia and unfertilised) a more systematic approach is needed to enumerate the baseline of egg losses and to test techniques to reduce these losses.
- Continuation of the feed trials to determine the relationship between the first adult moult and cannibalism versus starvation period.

As this is the first time in crayfish history that the use of large quantities of craylings can be utilised in this manner, the transport, handling and husbandry of them will need to be investigated further. For example, currently 20 000 crayling have been transported in one standard foam seafood box over 28 hours without mortality, however the maximum time limit is not yet known.
Another question is how to best prepare new ponds to ensure the optimum survival of craylings. Feedback from experienced growers who have used craylings are beginning to provide valuable insight into avenues for improving crayling husbandry.

10. Conclusion

A wide variety of bacteria have been cultured from all the samples that have been collected. Furthermore, there does not seem to be any pattern of bacterial growth. These facts make coming to any conclusions difficult; however, the following could be ascertained:

1. The tests from the API 20E system were used and the results compared to tables compiled from published identification tables for *Aeromonas* and pseudomonads originating from the Journal of International Systematic and Evolutionary Microbiology (Figueras et al. 2011, Martínez-Murcia, et al. 2008) . The primary sources of the data in the tables were obtained from various publications in the Journal of International Systemic and Evolutionary Microbiology. Identification of bacteria within this group was found to be adequate and in agreement with the PCR results. The Biolog Aerobic Bacterial Identification System purchased by the School of Veterinary and Biomedical Sciences has improved the identification of the bacteria. In fact, it was more discriminatory than both the API 20E identification system and PCR sequencing using the selected genes.
2. The presence of bacteria together with an inflammatory response in the hepatopancreas is usually indicative of a bacterial septicaemia.
3. The predominance of either *Chromobacterium* (March 2012) or *Aeromonas hydrophila* (November 2012 and December 2012) in the larvae and egg samples coincided with periods of high mortality in the stage 2 larvae that had histological evidence of a Gram-negative septicaemia.
4. The role of the Gram-negative coccus *Acinetobacter* genospecies 6 is uncertain as it seemed to have entered the hatchery late in the hatchery cycle. Since it was also cultured from unhealthy larvae, its role in the disease process cannot be excluded.
5. The positive effect of the commercially available probiotic *Bacillus* species seems to be minimal. It did not inhibit the development of the *Chromobacterium* or *A. hydrophila*-associated bacterial septicaemic disease in the stage 2 larvae in March, November and December 2012. It was not shown to produce any beneficial antibacterial substances. The only possible positive effect is to provide a biofilm in the hatchery prior to colonisation by other bacteria. In spite of this, *A. hydrophila* was still able to grow and produce more dominant biofilms.

6. Although fungi are occasionally noted to be a problem in the hatchery, the lack of high numbers of fungi and the absence of *Saprolegnia* indicate that the control measures are adequate to prevent fungal infections. However, certain *Fusarium* species may at times cause disease. Therefore, it is critical to be constantly vigilant and keep measures in place to both monitor and exclude fungi. Control measures included the use of dehumidifiers in the hatchery to keep roof, walls and air free of high moisture levels containing spread of water borne fungi and spores.
7. To date this project failed to yield a non-pathogenic probiotic. *A. hydrophila* was only present in appreciable numbers when the mortality rate of the S2L in the hatchery was high. The trials to determine whether other bacteria could out-compete *A. hydrophila* indicate that so far none of them can.
8. The testing of other bacteria will continue indefinitely in attempts to identify potential probiotics with inhibitory effects on pathogenic species. Currently 3 species of non pathogenic aeromonad are being trialled.
9. *Acinetobacter* genospecies 6 (A), *Acinetobacter grimontii* (B), *Chryseobacterium balustinum* (C) were found under ideal growth conditions not to be able to out-compete *A. hydrophila*. This is indicative that they may not be good probiotics.
10. Some isolates in the *Pseudomonas putida* group could yet become probiotic candidates. They were not further characterised as they were only isolated in large numbers in the August sampling and were isolated in low numbers together with *Chromobacterium*, indicating that they may have little effect. Nevertheless, they were isolated from the hatchery when it was functioning well and they are fast growers that are not known to cause disease.
11. S2L are able to obtain nutrition from both the egg yolk and from bacteria in biofilms while S3J's with the depletion of egg yolk, rely less on bacteria, and have turned to other available sources of nutrition such as the exoskeleton from moults or cannibalism. Therefore, theS3J craylings would benefit from being fed as soon as possible within the 14 day period tested in the feeding trial.
12. The survivability of S3J craylings was much higher when fed than when not fed.
13. The feeding trial results indicate that S3J craylings should be fed, but it may not be necessary to feed them before they are 14 days of age.

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12. Appendix

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