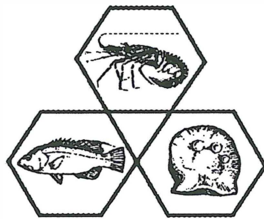
Control and Detection of Microsporidiosis in Freshwater Crayfish

J.S. Langdon
&
T. Thorne



FISHERIES DEPARTMENT
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Project 90/78

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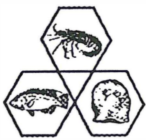

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Control and detection of microsporidiosis in freshwater crayfish

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Fish Health Section

Fisheries Department of Western Australia

c/o Animal Health Laboratories

WA Department of Agriculture

Baron-Hay Court

South Perth WA 6151

Fishing Industry Research and Development Council

SUMMARY

Microsporidiosis is a disease of freshwater crayfish and other crustacean species that has been noted as a significant problem in the farming of these animals. The condition is caused by a spore-forming protozoan parasite that has become known as "cotton-tail" or "porcelain" disease. The parasite can infiltrate muscle tissue resulting in flesh spoilage and in advanced cases, may lead to death.

Prevention, control and detection of the disease has been difficult to achieve, due to a lack of information on the parasite's life-cycle. This study was conducted to evaluate the methods of transmission of the parasite, ways of breaking the life-cycle, to evaluate the use of chemicals to prevent transmission and to evaluate detection methods for microsporidiosis. Results generated as a consequence of this project are listed below:-

As a consequence of this project, a very effective, highly sensitive spore concentration method was developed. The technique has a predictive value of>99% and will prove useful to Fish Health Laboratories for detecting microsporidiosis in crayfish destined for translocation.

Experimental transmission of the Western Australian microsporidian *Vavraia parastacida* by various methods, to marron, *Cherax tenuimanus*, yabbies, *Cherax albidus*, redclaw *Cherax quadricarinatus* and gilgies, *Cherax quinquecarinatus* was attempted with this study. Direct feeding of infected muscle resulted in transmission of the disease to juveniles of marron, yabbies and redclaw, although marron and gilgies may be refractory to infection after they reach maturity. In general, yabbies appear to be more susceptible and showed a much more rapid and severe progress of the disease than marron, with lesions visible after 2 months and death after 4-6 months. This rapid progress could be explained by their status as novel hosts, and may also explain why natural infections have not yet been detected. It cannot be emphasised strongly enough that the susceptibility of yabbies, originally from Victoria, to a novel microsporidian from WA, may be no less than the susceptibility of WA marron to *Thelohania* from yabbies in the east.

Fumagillin, the anti-microsporidial drug tested in this project, did not prevent or ameliorate infections in either marron or yabbies and as the unregistered drug was ineffective, its use is not recommended. General opinion is now held that as few, if any, drugs of this nature are registered for use in animals destined for human consumption their use could be detrimental to the freshwater crayfish industry as a whole.

Results on the life-cycle of the parasite and the need for an intermediate host were inconclusive and varied with the species. No transmission was obtained using a series of possible intermediate hosts, and the effect of priming chemicals on the spores produced varied results.

BACKGROUND

The farming of the Australian freshwater crayfish yabbies (*Cherax albidus*), marron (*Cherax tenuimanus*) and redclaw (*Cherax quadricarinatus*) is a small but rapidly expanding industry over most states of Australia. Microsporidiosis is a disease of freshwater crayfish that has been noted as a significant problem in the farming of these animals, although the industry is relatively free of other diseases of major concern.

Microsporidiosis is commonly known as "cotton-tail" or "porcelain disease" and is caused by the microsporidian protozoans *Thelohania*, *Pleistophora* and related genera. *Thelohania* and *Thelohania*-like species occur in yabbies in South Australia (P. O'Donoghue pers. comm.), in Victoria and New South Wales (Carstairs 1978), gilgies (*Cherax quinquecarinatus*) in Western Australia (L. Evans pers. comm.) and redclaw marron in Queensland (Herbert 1988). A *Pleistophora*-like species has been reported from yabbies from South Australia (O'Donoghue et al 1990), and we have encountered and described a new microsporidian species, *Vavraia parastacida*, in marron and gilgies and in redclaw imported from Queensland (Langdon 1991a, 1991b).

Currently, thelohaniasis in yabbies is the only microsporidiosis causing large losses of freshwater crayfish. The infection occurs predominantly in locomotor, cardiac and gastro-intestinal striated muscle resulting in flesh spoilage and eventually death (Carstairs 1978; Herbert 1988). *Vavraia parasticida* in marron causes progressive infiltration and inflammation of locomotor and gastric muscle, and the gastric lesions appear to be the cause of death in advanced cases.

No effective treatments are available for microsporidiosis and development of any treatment or control measures has been hampered by a lack of information on the parasite's life-cycle. Most workers consider direct transmission between crayfish to be unlikely. Herbert (1988) was unable to induce transmission of a *Thelohania* species in redclaw by feeding the spores directly or via fish. Iverson and Kelly (1978) showed that passage of *Thelohania* spores through the gut of fish primed them for subsequent infection of prawns, but other routes of infection were not completely excluded. It is possible to prime spores of some insect microsporidians and make them infectious by treatment with chemicals such as potassium chloride, potassium hydroxide and hydrogen peroxide (Sweeney, Hazard and Graham 1985). Evidence does suggest that invertebrate intermediate hosts, such as simuliid blackfly larvae, can play a role in transmission of microsporidiosis to crayfish (Chartier and Chaisemartin 1982).

Control of microsporidiosis by the use of chemicals such as monensin, benomyl and fumagillin have been investigated and appear to be effective in suppressing the pre-spore stages of microsporidian development (Overstreet 1988). Although these chemicals are not registered for use in food animals, they may be useful in suppression of vegetative stages in broodstock, allowing production of disease-free progeny.

Early microsporidian infections are hard to detect in crayfish, before development of a

visible lesion. For health certification and effective control programmes there is a need to increase the efficiency and level of confidence required to reliably detect the disease. We have developed a spore concentration method that greatly increases the chance of detecting sub-clinical infections in gills and muscles of crayfish.

NEED

1. As very little is known of the life-cycle of microsporidians in general, it would be of great benefit to crayfish farmers to better understand the mode of transmission of the disease in the attempt to control and prevent the spread. Identifying intermediate or priming hosts would aid in the prevention of the disease, by excluding such vectors from crayfish farms. Although chemotherapy appears to be of little use in established infections, it may be of benefit in suppressing the pre-spore stages of microsporidian development.

2. Detection of microsporidosis is difficult in early infections before visible lesions occur, but vital to effective control programmes and health certification of disease-free stocks for use by farmers. So an effective, highly predictive technique is required to detect this disease.

OBJECTIVES

1. To determine the modes of transmission for microsporidiosis affecting freshwater crayfish.

These, and previous studies (Langdon and Thorne 1992) show that *Vavraia* is effectively and directly transmitted by ingestion of infected muscle, and that both marron and yabbies can be infected by this route. This is not surprising given the common occurrence of cannibalism in crayfish populations. As crayfish are probably the only hosts involved, movement of infected animals is likely to result in the spread of disease to the site of introduction.

2. To evaluate the use of chemicals to prevent transmission of critical life-cycle stages.

Experiments involving fumagillin treatment of the parasite, by feeding infected muscle or pellets, soaked in fumagillin, did not prevent infection. Similarly, adult infected marron show no amelioration of established infection when fed fumagillin directly. It appears therefore, that fumagillin is of no use as an anti-microsporidial drug and as the drug is not registered for use on animals destined for human consumption, its use would not be recommended.

3. To formulate methods for breaking the life-cycles of microsporidians on crayfish farms.

Direct transmission appeared to be the most likely means of infection in this study and this may be minimised by provision of shelter for freshly moulted and young crayfish to prevent cannibalism. As the results on determining intermediate or priming hosts were inconclusive,

methods to prevent cannibalism and culling of infected stock appear to be the only effective method of breaking the life-cycle of this disease at this stage.

4. To evaluate detection methods for microsporidiosis.

A highly effective spore concentration method (>99% predictive value) has been developed and reported in Langdon (1991a), Langdon and Thorne (1992), and details are given in the Continuing Grant Application Report of 1992/1993. This work also resulted in a study on the detection of *V. parastacida* by Ostle (1991).

RESEARCH METHODOLOGY

To achieve objectives 1-3 required experimental transmission of *V. parastacida* under a variety of conditions, and the parameters tested were target species (marron, yabbies, gilgies, redclaw, *Gambusia*), age of targets, cultured parasites against natural infections, feeding parasites treated with anti-microsporidian drugs (fumagillin), feeding possible intermediate hosts from naturally infected sites, and feeding parasites pre-treated by exposure to priming chemicals (potassium hydroxide and hydrogen peroxide).

Details of these techniques have been published in Langdon (1991a, 1991b, 1992) (attached).

DETAILED RESULTS

Some of the results of transmission experiments have been published in Langdon (1992). In all cases where results are given, replicate controls were negative for infection.

Host species and age

The results of feeding different species of crayfish of different ages *per os* with spores concentrated from natural infections are given in Table 1. All feeding trials using marron under the age of 12 months examined several months after feeding, resulted in some being infected. Two month old redclaw fed *per os* also become infected. Adult gilgies and *Gambusia* fed infected crayfish muscle showed no subsequent signs of infection. However, 6 week old and 1 mo yabbies became 67% and 22% infected respectively. It is concluded that yabbies are more susceptible to the parasite than marron, and that adult gilgies and adult marron may be refractory to infection.

Cultured and natural parasites fed *per os*

Two out of four 3 mo marron fed purified spores in phosphate buffered saline and antibiotics (penicillin and streptomycin) and examined 4.5 months later were infected, but none of eight 4 mo marron fed spores cultured and passaged twice on a mosquito epithelial cell-line (C6-36) became infected. Subsequent transmission electron microscopy of intracellular spores in culture showed them to be of normal appearance, although these may have been the original

spores used to infect the cell-line, that had not developed at all. However, failure to infect may have been due to the slightly older marron used rather than cultured spore viability. Eighteen 1 mo yabbies were fed spores cultured in and passaged three times on C6-36 cell lines, of which 3 were infected 6 months later. Replicate controls were fed only tissue culture media and all remained uninfected.

The effects of anti-microsporidian drugs

Fumagillin was chosen as the anti-microsporidian drug which is active against spores. Three out of nineteen 6 mo marron fed per os with infected muscle, and then for 2 weeks with 0.5% fumagillin treated pellets became infected with the parasite when examined 4.5 months later. Although, one out of eight 6 mo marron simply fed infected muscle, without subsequent fumagillin treatment, also become infected. Similarly, eight out of twenty 6 mo marron fed infected muscle soaked in 2.5% fumagillin became infected after 4.5 months, and 5 out of 18 of the control group of marron subject to the same protocol, but without fumagillin, became infected. It therefore seems that in marron it is possible that fumagillin may potentiate the infectivity of the parasite, possibly by suppressing the host immune response, although statistical analysis of this data has not been performed to confirm this result.

Trials involving the feeding *per os* of infected muscle to 1mo yabbies who where subsequently fed feed containing 0.5% fumagillin for 2 weeks, did not show any controlling effect as the animals became infected at the same rate as the positive controls.

Observations on possible intermediate hosts

Tubificid worms (*Tubifex* sp.), which are common in rivers, were fed infected muscle and examined 1 mo later, but there was no sign of microsporidian stages present in organ smears. More infected muscle and *Gambusia*, pond detritus and sediment and cladocerans were added to the tank, and 4 months later forty 1 mo yabbies were added. When examined 7.5 months later, none of six yabbies examined were infected. In another trial, blackfly larvae (*Simulium* sp.) from the Helena River, WA, were fed infected muscle and examined one week later for microsporidian infections, but none were observed.

Effects of priming chemicals

Spores purified from 1, 2 or 3 passages in C6-36 cell-lines that were either treated with 10% hydrogen peroxide for 15 min at 4_SYMBOL 176 \f "Symbol"_C, or with 0.1M potassium hydroxide (KOH) for 65 min at 22 C, were fed to 3 mo marron. One of 6 marron fed KOH treated spores was infected, but none of 7 marron fed hydrogen peroxide treated spores were infected, after 4.5 months. In a similar experiment, purified spores were layered over rainbow trout gonad (RTG-2) cell-lines, passaged once or twice over 14 days, treated as above or left untreated, and fed to 3 mo marron. Upon examination 4.5 months later, none of 7 marron were found to be infective.

In another experiment, purified spores were passaged through C6-36 cells 1-3 times, treated as

above or left untreated and fed to 6 week old marron. After 4.5 months, 2 out of 15 marron were found to be infected.

DISCUSSION

The results generated from this project on the experimental transmission of *Vavraia parasticida* into marron and yabbies has been reported (Langdon and Thorne 1992). More details of the laboratory methodology used during the course of this project are documented in this paper.

The results of direct transmission studies using chips of muscle from natural infections which are reported here, suggest that marron less than 12 mo are susceptible to infection, but when >12 mo they become refractory to infection. From previous data (Langdon and Thorne 1992), it was observed that marron at >4 mo may be refractory to infection because they can mount a strong, but inadequate immune response. Trials outlined in this report indicate that only marron over 12 months of age are refractory to infection. Resistance to infection in >12 mo marron may be due to increased chitin deposition in the fore- and hind- gut of the host with age, changes in gut physiology, or a change in the immune response of the host allowing destruction of new infections after the host is 12 mo. The latter case may also explain why 6 mo marron treated with fumagillin for 2 weeks after exposure to *Vavraia*, or chips of muscle containing *Vavraia* were soaked for 24 hours in fumagillin before feeding, resulted in a slightly higher number of infected animals than the positive controls. Fumagillin does not appear to kill the parasite and may indeed suppress the immune response of 6 mo marron, making them slightly more susceptible to infection.

As marron >12 mo do not become experimentally infected with untreated natural spores, and progression of disease to an advanced state probably takes about one year (Langdon 1991a, Langdon and Thorne 1992), it would be expected that moribund marron >16 mo would be rarely encountered. However, natural infections have be found in marron of about 5 years old. These infections in old hosts may represent attenuation of a chronic infection that occurred in the first few months after hatching, or may represent a separate infection event. Interestingly, lesions occur predominantly in the dorsal muscle of the anterior third of the tail in experimental animals (Langdon and Thorne 1992) but are usually in the locomotor, cardiac and gastrointestinal striated muscle in natural infections (Langdon 1991a). This may also be interpreted as due to two different infection processes.

Differences in infection may be due to the utilisation of an intermediate host, and infection by different stages of the parasite. There have been several reports of mosquito microsporidians utilising a second host (Andreadis 1990, Avery and Undeen 1990, Sweeney et al. 1985, 1990) in which they can undergo complex and often plastic developmental cycles (Andreadis 1985, Avery and Undeen 1990) involving 3 sporulation sequences, two in mosquitoes and one in the copepod (Sweeney et al. 1988), and 2-3 types of spore (Avery and Undeen 1990, Dickson and Barr 1990). However, although microsporidians infect all

metazoan groups and have been subject to intensive study, intermediate hosts are only know for mosquito microsporidians. Also, horizontal infection cycles are always initiated by uninucleate spores, and binucleate spores are only involved in vertical transmission within the mosquito population. Therefore secondary hosts are unusual, and there is no evidence for transmission other than by spores.

Experimental attempts to utilises other hosts or cell-lines gave negative results, except for the apparent positive results in one trial using the C6-36 cell-line. However that result must be discounted, as infection may have been effected by parasite stages originally used to infect the cell-line. As secondary hosts are unknown for microsporidia, except for those parasitising mosquitoes, and spores are the only known infective stage, infection of adult marron is probably due to cannibalism, as in young (<12 mo) crayfish. Infection of adult marron is very uncommon, and may be due to environmental stress and/or immune suppression, with environmental stress probably causing immune suppression. The only heavily infected adult marron found in WA rivers have been from the Hotham river/Williams river system, which has had increasing eutrophication and declining marron populations for several years (Noel Morrissy: pers. comm.). Microsporidiosis due to *Thelohania contejeani* increases in crayfish (*Orconectes virilis*) living in Canadian lakes that have become acidified, and this has been attributed to decreased host resistance when under sublethal stress (France and Graham 1985).

The results obtained using similar direct feeding regimes to young yabbies suggest that they are more susceptible to infection. Yabbies also show a much more rapid and severe progress of disease, with lesions visible after 2 months, clinical illness by 4 months and death after 4-6 months, and this has been attributed to their status as novel hosts that have not evolved immunocompetance (Langdon and Thorne 1992). The rapid progress of disease may explain why natural infections have not been observed in yabbies, but given the difference between infection patterns in experimentally infected and in wild marron, such an explanation should be treated cautiously. It cannot be emphasised strongly enough that the susceptibility of yabbies, originally from Victoria, to a novel microsporidian from WA, may be no less than the susceptibility of WA marron to *Thelohania* from yabbies in the east.

It is possible that yabbies fed C6-36 cell lines that subsequently became infected, may have been infected by the material originally used to infect the cell-lines. Electron micrographs of infected C6-36 cells (an epithelial cell-line) show intracellular spores in vacuoles resembling phagosomes rather than parasitophorous vacuoles. Also similarly to the results on marron, the attempts at infection of yabbies using *Tubifex* fed infected meat, pond detritus, etc, cannot be taken to preclude the need for factors to trigger spore infectivity.

The studies undertaken under this grant have shown that marron and yabbies can become infected with *Vavraia parasticida* by direct transmission, and that yabbies appear more susceptible to infection. The severity of the disease in yabbies, a novel host, compared with marron, the normal host of *Vavraia*, highlights the likely outcome for marron if yabbies infected with *Thelohania* sp. were to be introduced into WA from the eastern states. There is a very real danger for both marron farming, from *Thelohania* and yabby farming, from *Vavraia* in WA.

It appears that although only young marron and yabbies are susceptible to infection, adult marron, and probably adult yabbies, may have increased susceptibility to disease when stressed or immunosuppressed. If so, poor management practices that stress stock on crayfish farms with sub-clinical levels of disease may result in outbreaks of disease. Similarly decline in the quality of water in natural systems is likely to result in increased incidence of microsporidiosis.

BENEFITS

There are three main beneficiaries of the results stated in this study. Fish farmers, Fish Health Laboratories and Fisheries Management Authorities. Adoption of the recommendations highlighted in this report will enable fish farmers to formulate effective control and prevention measures on farms, Fish Health Laboratories to detect low level infections of microsporidial disease in the laboratory and Fisheries Management Authorities to devise policies on the movement of freshwater crayfish in and around the country.

STAFF

Dr. J.S. Langdon, Senior Fish Pathologist

Ms. T.J. Thorne, Technical Officer

Dr. P.M. Hine, Senior Fish Pathologist

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TABLE 1: Results of per os feeding of infected muscle to various crayfish species

Species	n (n final)	Age at treatment (months)	Age at termination (months)	#+ve
Cherax tenuimanus	30(6)	1	4.5	1/6
	10(5)	3	4.5	5/5
	16(8)	4	8	4/8
	25(8)	6	10.5	1/8
	25(18)	6	10.5	5/18
	5(5)	12	19	0/5
Cherax albidus	50(23)	1	7	5/23
	200(12)	1.5	12.5	8/12
	25(18)	6	11	3/18
Cherax quinquecarinatus	14(11)	adult	7	0/11
Cherax quadricarinatus	20(15)	2	9	3/15