Vol. 43: 127–137, 2000 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published November 14

Luminous vibriosis in rock lobster *Jasus verreauxi* (Decapoda: Palinuridae) phyllosoma larvae associated with infection by *Vibrio harveyi*

B. K. Diggles[1,*], G. A. Moss[1], J. Carson[2], C. D. Anderson[3]

[1]National Institute of Water and Atmospheric Research Ltd., PO Box 14-901, Kilbirnie, Wellington, New Zealand
[2]Department of Primary Industries, Water & Environment, PO Box 46, Kings Meadows, Launceston, Tasmania 7249, Australia
[3]National Centre for Disease Investigation, Ministry for Agriculture and Forestry, PO Box 40742, Upper Hutt, New Zealand

ABSTRACT: Studies were conducted to determine the cause of outbreaks of luminous vibriosis in phyllosoma larvae of the packhorse rock lobster *Jasus verreauxi* reared in an experimental culture facility. On 2 separate occasions mortalities of up to 75% over a period of 4 wk were observed in 4th to 5th and 8th to 10th instar phyllosomas at water temperatures of 20 and 23°C, respectively. Affected larvae became opaque, exhibited small red spots throughout the body and pereiopods, and were faintly luminous when viewed in the dark. Histopathology showed that the gut and hepatopancreas tubules of moribund phyllosomas contained massive bacterial plaques. The hepatopancreas tubules of moribund larvae were atrophic and some contained necrotic cells sloughed into the lumen. Dense, pure cultures of a bacterium identified as *Vibrio harveyi* were isolated from moribund larvae. The disease syndrome was reproduced by *in vivo* challenge and *V. harveyi* was successfully reisolated from diseased larvae after apparently healthy larvae were exposed by immersion to baths of more than 10^4 *V. harveyi* ml^{-1} at 24°C. Injured larvae were more susceptible to infection than were healthy larvae. Survival of larvae experimentally and naturally exposed to *V. harveyi* was improved when antibiotics were administered via bath exposures.

KEY WORDS: Spiny lobsters · Crustacea · Mariculture · Disease · *Vibrio* · New Zealand

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Disease management is important when maintaining lobsters in captivity (Fisher et al. 1978, Stewart 1980, 1984, Sindermann 1990). Diseases which have caused economic losses in captive clawed lobsters (Decapoda: Nephropidae) include bacterial infections, particularly shell disease and gaffkemia (Stewart 1984), fungi (Unestam 1973, Fisher et al. 1975, 1978), protozoa (Cawthorne et al. 1996), and nutritionally related diseases such as moult death syndrome (Bowser & Rosemark 1981). In contrast, relatively few diseases have been reported to date from captive spiny lobsters (Decapoda: Palinuridae) (see Evans & Brock 1994). The high commercial value of spiny lobsters has resulted in interest in their aquaculture (Booth & Kittaka 1994). There may be considerable potential to increase spiny lobster production through rearing lobsters from eggs (Kittaka 1994, 1997, Kittaka et al. 1997) or growout of pueruli collected from the wild (Booth & Kittaka 1994, Hooker et al. 1997). As the number of instances where spiny lobsters are held in captivity at high density increases, a concurrent increase in reports of their diseases may be expected. This paper reports the first record of *Vibrio harveyi* from cultured lobsters in New Zealand and describes 2 outbreaks of luminous vibriosis in phyllosoma larvae of the packhorse rock lobster *Jasus verreauxi* (Milne Edwards 1851).

*E-mail: b.diggles@niwa.cri.nz

© Inter-Research 2000

MATERIALS AND METHODS

Larval rearing. Phyllosomas of *Jasus verreauxi* were reared for over 100 d at 19°C in an upwelling tank system (Illingworth et al. 1997) at the Mahanga Bay Aquaculture Research Facility (Wellington, New Zealand) using partial seawater recirculation at 150% replacement d^{-1}. Seawater was filtered through diatomaceous earth to 1 µm and UV-irradiated. Tanks were maintained at light levels below 0.001 µmol s^{-1} m^{-2} (Moss et al. 1999). Phyllosomas were fed 5 to 7 d old, 2 to 3 mm long brine shrimp *Artemia salina* and had reached instars 8 to 10 (Kittaka et al. 1997). The same methods were being used 12 mo later prior to a second disease outbreak in 4th to 5th instar phyllosomas, except the water temperature was maintained at 20°C.

Bacteriology. During the original disease outbreak, 4 moribund *Jasus verreauxi* phyllosoma larvae exhibiting clinical signs of disease and 3 apparently healthy larvae were surface-disinfected by immersion in 70% ethanol for approximately 10 s. The surface-sterilised larvae were held in flame-sterilised forceps and blotted on lint-free tissues to remove excess ethanol before being sagittally sectioned with flame sterilised microscissors. The cut surface of one side of each larva was used to inoculate plates of thiosulphate citrate bile salt sucrose agar (TCBS, Oxoid), and tryptic soy agar with 2% NaCl (TSA+2) (Gibco BRL) which were incubated at 20°C and examined daily for 7 d. Bacteria isolates were subcultured to ensure purity before being stored in long-term preservation medium (Beuchat 1974) for up to 1 wk prior to identification. Biochemical characterisation was undertaken by subjecting isolates to 52 phenotypic tests using methods described by Baumann et al. (1971), Furniss et al. (1978), and West & Colwell (1984). Luminescence was assessed by growing bacteria on plates of Schneider & Rheinheimmer's Medium (Austin & Lee 1992) and incubating the cultures at 15°C for 24 h. Plates were examined in total darkness for the presence of luminous colonies. Phenotypic data were compared to a probabilistic data matrix for *Vibrio* species (Bryant et al. 1986) using a regularly updated version of the Bacterial Identifier program (Bryant & Smith 1991). An acceptable identification was reached when the identification score equaled or exceeded 0.98. Identical methods were used to identify bacteria isolated during the second disease outbreak.

Antimicrobial sensitivities. Minimum Inhibitory Concentration (MIC) tests were performed only on the *Vibrio harveyi* isolates obtained during the original disease outbreak. Tests for 14 antibiotics (Sigma-Aldrich), including oxolinic acid, oxytetracycline, chloramphenicol, trimethoprim, nitrofurantoin, furazolidone, neomycin, ampicillin, amoxycillin, co-trimazine (trimethoprim/sulphadiazine [1/5]), sulphadiazine, sulphamethazine, streptomycin and trimethoprim/sulphamethazine (1/5), were based on the NCCLS microbroth dilution procedure as described by Carson & Statham (1993). Stock concentrations of 0.1% w/v, active ingredient, for each antibiotic were prepared using the solvents described by Anhalt & Washington (1991) and diluted further with Mueller-Hinton broth (MHBS) (Oxoid) supplemented with NaCl to a final concentration of 1% w/v. Doubling dilutions of the antibiotic in MHBS were prepared in sterile microtitre trays (Nunc) to give a range of final antibiotic concentrations between 25 and 0.02 µg ml^{-1}. The broths were inoculated with *V. harveyi* harvested from an overnight plate culture and suspended in MHBS to a concentration equivalent to McFarland standard 0.5 as determined with a Hach 2001P Turbidimeter. The plates were incubated at 25°C for 24 h and MIC endpoints identified by visual inspection for the presence/absence of growth.

Histology. The unused halves of the sagittally sectioned phyllosoma larvae (4 moribund and 3 apparently healthy individuals) examined during the original disease outbreak were immediately fixed in 10% (v/v) formalin in seawater filtered to 0.22 µm. All samples were embedded in paraffin wax for sagittal sectioning (6 µm) using standard histological procedures, stained with hematoxylin and eosin and examined using light microscopy.

Experimental infections. ***Trial 1:*** Bacteria used for experimental infection of healthy larvae were harvested from the edge of *Vibrio harveyi* colonies subcultured on TCBS at 20°C from isolates obtained from the original outbreak of luminous vibriosis. The bacteria were suspended in 10 ml of seawater sterilised by filtration (0.22 µm pore size) and a 10-fold dilution series in filter sterilised seawater was prepared from 10^{-1} to 10^{-3} in sterile 15 ml centrifuge tubes. An estimate of the cell number in the original suspension was made by direct microscopic count of motile bacteria in the 10^{-3} dilution using a Reichert Brite-line Improved Neubauer hemocytometer. The mean of 5 replicate counts was used. Final concentrations used in the experimental beakers were prepared by adding appropriate volumes of a stock suspension of approximately 10^8 bacteria ml^{-1} *V. harveyi*. Groups of 5 apparently healthy *Jasus verreauxi* phyllosomas (instar 8 to 10) were placed in six 1 l glass beakers in a 24°C water bath. Each beaker contained 400 ml of UV-irradiated, filtered (1 µm pore size) seawater. The phyllosomas in Beakers A, B and C were exposed to *V. harveyi* at concentrations of 10^4, 10^6, or 10^7 *V. harveyi* ml^{-1}, respectively, by direct immersion (Table 1). The remaining 3 beakers (D, E, and F) were negative controls and had no bacteria added. After 48 h the water in all beakers was diluted 50% by the addition of 400 ml of filtered (1 µm pore size) and UV-irradiated seawater. The trial was terminated 7 d after

Table 1. Details of experimental design and results of experimental challenges Trial 1 of healthy *Jasus verreauxi* larvae (8th to 10th instar) exposed to *Vibrio harveyi* by bath immersion or via *Artemia* at 24°C. –: no attempt made to reisolate bacteria from non-luminescent phyllosomas

Beaker	Bacterial concentration (ml^{-1})	Method of exposure	No. of larvae	Feeding details	% survival after 7 d	*V. harveyi* reisolated
A	10^4	Bath	5	Unfed	40	From 100% of dead larvae
B	10^6	Bath	5	Unfed	80[a]	–
C	10^7	Bath	5	Unfed	0	From 100% of dead larvae
D	0	Control	5	Unfed	100	–
E	0	Control	5	Fed *Artemia*	100	–
F	0	Control	5	Unfed	100	–

[a]1 phyllosoma unaccounted for

the initial challenge with *V. harveyi*. The number of dead or luminous phyllosomas was noted for each treatment. The phyllosomas in Beakers A, B, C, D and F were not fed during the experiment; however control phyllosomas in 1 beaker (Beaker E) were fed with *Artemia* to assess whether mortalities could be attributed to not feeding larvae for 7 d (Table 1). Water temperature remained at 24°C and salinity 35‰ throughout the experiment, which was conducted under a 24 h dark photoperiod. The water in all beakers was not aerated for the duration of this experiment.

Trial 2: A second experiment was performed 11 mo after the original disease outbreak to determine if injured larvae were more susceptible to infection by *Vibrio harveyi* during bath exposures. Groups of 10 apparently healthy *Jasus verreauxi* phyllosomas (instar 4 to 5) were each placed in four 1 l glass beakers containing 1000 ml of filtered (1 µm pore size), UV-irradiated seawater. All of the phyllosomas in Beakers A and B had 2 pereiopods severed using flame sterilised microscissors, while all phyllosomas in Beakers C and D were left intact. Approximately 10^7 colony forming units (CFU) of *V. harveyi* in 1 ml of tryptic soya broth (TSB) (Oxoid) was then added to Beakers B and D to make a bath concentration of 10^4 CFU ml^{-1}. The *V. harveyi* used in this experiment was recovered from 11 mo storage in long-term preservation medium and cultured for 24 h at 20°C in TSB. The number of *V. harveyi* CFU ml^{-1} of TSB used to inoculate beakers was determined using a 10-fold dilution series in TSB from 10^{-1} to 10^{-5}. A 0.1 ml aliquot of the 10^{-5} dilution was plated out on TSA+2 and colonies were counted after 18 h incubation at 20°C. All beakers were examined daily and the trial was terminated 7 d after the initial challenge with *V. harveyi*. Water temperature remained at 22°C and salinity 35‰ throughout the experiment, which was conducted under a 24 h dark photoperiod. The water in all beakers was aerated and the phyllosomas were not fed for the duration of the experiment.

In vivo* antibiotic trials. *Trial 3: Groups of 5 apparently healthy *Jasus verreauxi* phyllosomas (instar 8 to 10) were placed in six 1 l glass beakers containing 400 ml of filtered (1 µm pore size), UV-irradiated seawater (Table 2) in a 24°C water bath. A bacterial suspension prepared as for Trial 1 was added to Beakers B, C, D, E and F to achieve a concentration of 5×10^6 *Vibrio harveyi* ml^{-1}. Twenty-four hours after introduction of the bacteria 4 antibiotic treatments, namely two 1:5 combinations of trimethoprim and sulphamethazine (Sigma-Aldrich) at 20:100 mg l^{-1} and 10:50 mg

Table 2. Details of experimental design and results for *in vivo* antibiotic Trial 3 on *Jasus verreauxi* larvae (8th to 10th instar) exposed by immersion to baths of 5×10^6 *Vibrio harveyi* ml^{-1} at 24°C. –: no attempt made to reisolate bacteria from non-luminescent phyllosomas

Beaker	Bacterial concentration (ml^{-1})	No. of larvae	Antibiotic treatment 24 h post exposure	% survival after 7 d	*V. harveyi* reisolated
A	0	5	None, control	100	–
B	5×10^6	5	None, control	60	No[a]
C	5×10^6	5	120 mg l^{-1} trimethoprim/sulphamethazine (1/5)	100	–
D	5×10^6	5	60 mg l^{-1} trimethoprim/sulphamethazine (1/5)	80	From 100% of dead larvae
E	5×10^6	5	0.1 mg l^{-1} nifurpirinol (prefuran)	20	From 100% of dead larvae
F	5×10^6	5	100 mg l^{-1} oxytetracycline hydrochloride	80	No[b]

[a]Phyllosoma with clinical signs of *V. harveyi* infection cannibalised before samples could be taken
[b]Affected phyllosoma died during a moult and exhibited signs of deformities

l^{-1}, nifurpirinol (0.1 mg l^{-1}) (Argent laboratories) and oxytetracycline hydrochloride (100 mg l^{-1}) (Sigma-Aldrich) were added to Beakers C, D, E and F, respectively (Table 2). Each antibiotic was dissolved separately in 100 ml of filtered (1 µm pore size), UV-irradiated seawater at 24°C and added to its respective beaker, with the beaker volume being made up to 800 ml with filtered and irradiated seawater. No antibiotics were added to Beakers A and B. Beaker A, which contained no bacteria or antibiotics, was used as a handling control, while Beaker B was used as a positive bacterial control. At the same time as antibiotics were introduced to Beakers C, D, E and F, the volume of Beakers A and B was increased to 800 ml by addition of 400 ml of filtered (1 µm pore size), UV-irradiated seawater. All beakers were examined daily for an additional 7 d. *Artemia* were added to all beakers as food on the Days 6 and 7 after addition of the antibiotics, and at the same time water changes of approximately 30% were performed. Water temperature remained at 24°C and salinity 35‰ throughout the experiment, which was conducted under a 24 h dark photoperiod. The water in all beakers was not aerated for the duration of this experiment.

Trial 4: A year after the original disease outbreak a second *in vivo* antibiotic trial was performed on *Jasus verreauxi* phyllosomas (instar 4 to 5) which were obtained from an upwelling system 24 h after mortalities due to luminous vibriosis were first observed. Groups of 10 *J. verreauxi* phyllosomas were each placed in sixteen 1 l glass beakers containing 1000 ml of filtered (1 µm pore size), UV-irradiated seawater. Half of the beakers were incubated in a water bath at 16°C and the other half were incubated at 20°C. The phyllosomas at each temperature were given either no treatment, or 10^4 CFU ml^{-1} *Vibrio harveyi* were added, 120 mg l^{-1} trimethoprim/sulphadimidine (1/5) added, or 10^4 CFU ml *V. harveyi* + 120 mg l^{-1} trimethoprim/sulphadimidine (1/5) added. Two replicate beakers were used for each treatment at each temperature. The number of *V. harveyi* CFU ml^{-1} was determined as described for Trial 2. All beakers were examined daily and the trial was terminated after 7 d. Salinity was 35‰ throughout the experiment, which was conducted under a 24 h dark photoperiod. The water in all beakers was aerated and the phyllosomas were not fed for the duration of this experiment.

Whenever dead phyllosomas were recorded in Trials 1 to 4, they were surface sterilised in 70% ethanol and samples were collected for bacterial culture and identification as described previously. Furthermore, all surviving phyllosomas were examined for bacteriology at the termination of Trials 2 and 4.

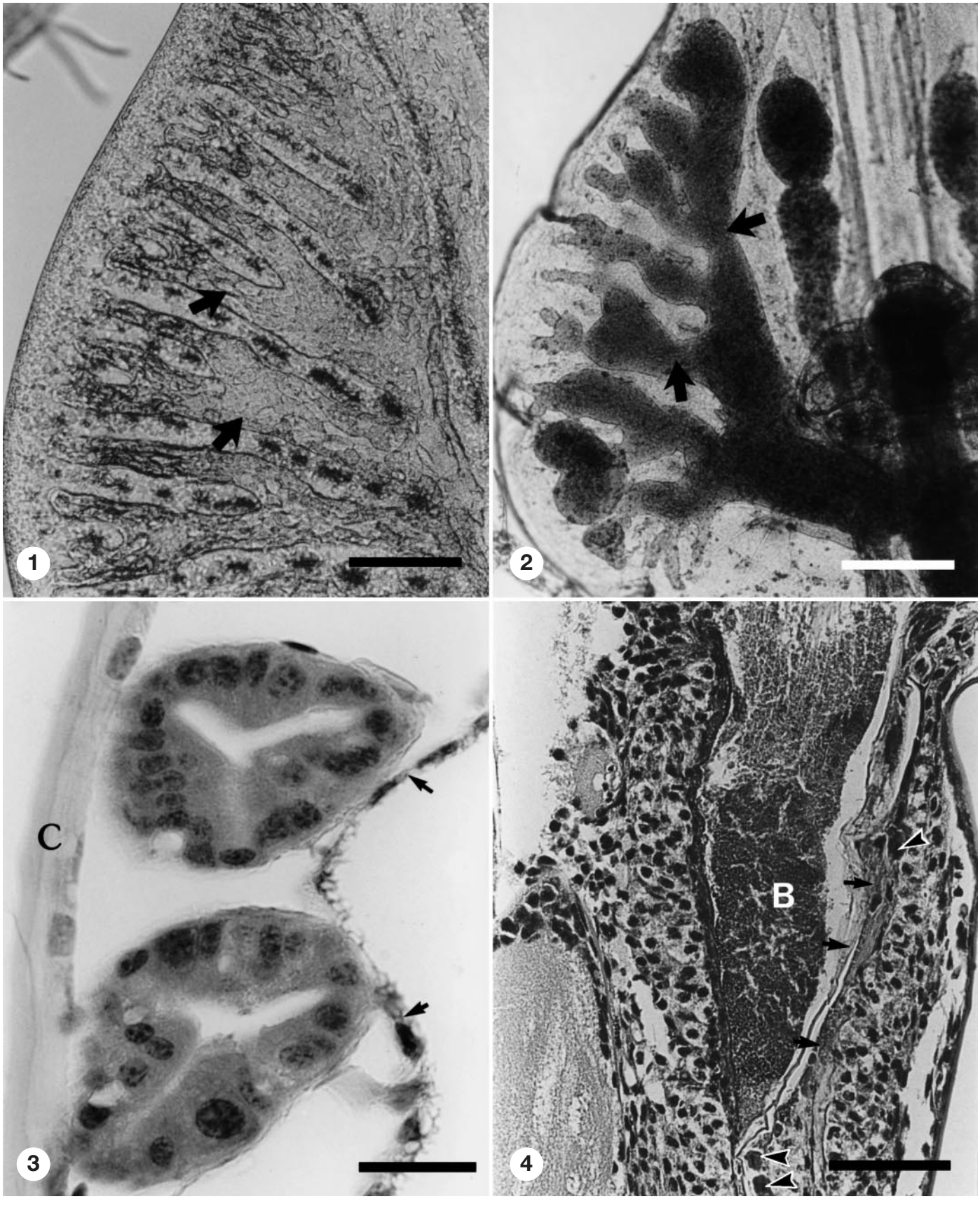
Statistics. The significance of differences in survival of larvae between treatments at the termination of Trial 4 was analysed from data pooled from individual beakers from both 16 and 20°C by 1-way analysis of variance using SigmaStat software (Jandel Scientific). Data were analysed for normality using the Kolmogorov-Smirnov test and differences between significant values were examined using the Student-Newman-Keuls method. Values of $p < 0.05$ were considered significant.

RESULTS

Onset of disease

During the original disease outbreak mortalities began in an upwelling tank containing approximately 850 phyllosoma larvae (instars 8, 9 and 10), 3 d after the water temperature was raised from 19 ± 1 to 23°C. Water temperature was reduced to 20°C immediately after the first mortalities were observed, but moderate mortalities (30 to 50 larvae d^{-1}) continued. Luminous larvae were first noticed 6 d after the water temperature increase. Apparently healthy phyllosomas were active and transparent when viewed in natural light (Fig. 1). Affected larvae were lethargic, slightly opaque when viewed in natural light (Fig. 2), exhibited small red spots throughout the body and pereiopods, and were faintly luminous when viewed in the dark. Mortality levels remained elevated for 2 wk, declined, then spiked sporadically until around 1 mo after the temperature change. After 1 mo, less than 200 larvae survived, representing a loss of over 75% of larvae during the disease episode. A second outbreak of luminous vibriosis occurred in the 4th to 5th instar phyllosomas around 12 mo after the original disease outbreak. This outbreak occurred at 20°C and did not follow an increase in water temperature. Clinical signs of disease and mortality rates were virtually identical to those observed during the original disease outbreak.

Figs. 1 to 4. *Jasus verreauxi.* Fig. 1. Gross appearance of the digestive gland of an apparently healthy phyllosoma viewed with transmitted light showing transparent appearance of tubules (arrows) which contain food material. Scale bar = 200 µm. Fig. 2. Gross appearance of the digestive gland of a luminous phyllosoma infected with *Vibrio harveyi*. Note opaque appearance of digestive gland tubules which are filled with bacteria (arrows). Scale bar = 200 µm. Fig. 3. Histology of hepatopancreas tubules of an apparently healthy phyllosoma. C: cuticle, arrows = connective tissue. Scale bar = 25 µm. Fig. 4. Histology of gut of a luminous phyllosoma. Note massive bacterial plaque (B), necrosis (arrows) and infiltrating haemocytes (arrowheads). Scale bar = 65 µm


1
2
C
3
B
4

Bacteriology

Heavy growth of a sucrose-negative (green coloured on TCBS) bacterium was isolated in pure culture on both TCBS and TSA+2 from 3 out of 4 of the moribund phyllosomas sampled during the original disease outbreak. No bacteria were isolated from the fourth moribund phyllosoma or from 2 of 3 apparently healthy larvae. A mixed culture of sucrose-positive colonies (yellow on TCBS) and sucrose-negative colonies was isolated from the third apparently healthy larva. All isolates obtained from moribund phyllosomas and the sucrose negative isolate from the apparently healthy phyllosoma were phenotypically identical (Table 3) and were identified unequivocally as *Vibrio harveyi* with an identification score of 0.98. These isolates gave atypical results for resistance to O/129 10 µg, alginase production, and utilisation of amygdalin, arbutin, L-leucine and D-glucosamine as sole carbon sources (Table 4). The remaining bacterium isolated in mixed culture from the apparently healthy phyllosoma was identified as a member of the genus *Vibrio*, but could not be identified to species (Table 3) and was not considered significant. The isolates are encoded under the Tasmania Department of Primary Industries, Water and Environment Fish Health Unit accession number 99/1052. Bacteria isolated from moribund larvae during the second outbreak of luminous vibriosis were again identified as *V. harveyi*; however, 4 of the 5 isolates examined during the second disease outbreak exhibited minor differences in their biochemical profiles compared to the original isolate. Test results which varied between these isolates included resistance to 10 µg of the vibriostatic agent O/129 (4 isolates), alginase negative (3 isolates), non-luminescent (2 isolates), acid production from sucrose (2 isolates), no acid production from mannitol (1 isolate), utilisation of sucrose (2 isolates), L-citrulline (1 isolate), and D-glucosamine (1 isolate) and inability to use L-leucine (3 isolates), amygdalin (3 isolates), D-glucuronate (2 isolates), and arbutin (2 isolates) as sole carbon sources.

Table 3. Biochemical characteristics of bacteria isolated from *Jasus verreauxi* larvae during the original disease outbreak. +: positive reaction, –: negative reaction, cs: carbon source, NT: not tested

Test	*V. harveyi*	*Vibrio* sp.
Swarming	–	–
Growth in 0% NaCl	–	–
Thornley's arginine dihydrolase	–	–
Moller's lysine decarboxylase	+	–
Moller's ornithine decarboxylase	+	–
Nitrate reduction	+	–
Oxidase	+	+
Indole	+	+
ONPG	+	–
Voges proskauer reaction	–	–
Resistance to O129 10 µg ml^{-1}	–	–
Resistance to O129 150 µg ml^{-1}	–	–
Resistance to ampicillin (10 µg)	+	+
Resistance to polymyxin B (50 i.u.)	–	–
Resistance to tellurite (0.0005%)	–	–
Aesculin hydrolysis	+	–
Alginase	+	+
Urease	+	–
Luminescence	+	NT
L-arabinose acid	–	–
Arbutin acid	+	–
Mannose acid	+	+
Salicin acid	+	–
Sorbitol acid	–	–
Sucrose acid	–	+
Mannitol acid	+	+
L-arabinose cs	–	+
Cellobiose cs	+	–
D-galactose cs	+	–
D-glucose cs	+	+
D-mannose cs	+	–
Melibiose cs	–	+
Lactose cs	–	+
Melizitose cs	–	+
Sucrose cs	–	+
Trehalose cs	+	+
Xylose cs	–	+
Ethanol cs	–	+
Glycerol cs	+	+
1-propanol cs	–	–
D-sorbitol cs	–	–
Gluconate cs	+	+
D-glucuronate cs	+	+
Amygdalin cs	+	+
Arbutin cs	+	+
L-citrulline cs	–	+
L-hydroxy proline cs	–	+
L-leucine cs	+	+
D-glucosamine cs	+	+
DL-3-hydroxybutyrate cs	–	+
a-ketoglutarate cs	+	+
Succinate cs	+	+

Antimicrobial sensitivities

In vitro tests found the *Vibrio harveyi* isolated during the original disease outbreak was most sensitive to oxolinic acid, with a MIC of 0.2 µg ml^{-1}, oxytetracy-

Table 4. Atypical phenotypic test results for isolates of *Vibrio harveyi* from *Jasus verreauxi* larvae during the original disease outbreak. cs: sole carbon source test

Test	Recorded result	% of strains positive for characteristic[a]
O/129 10 µg resistance	–	71
Alginase	+	38
Amygdalin cs	+	8
Arbutin cs	+	33
L-leucine cs	+	1
D-glucosamine cs	+	4

[a]51 *V. harveyi* strains in database

cline (MIC 0.78 µg ml^{-1}) and chloramphenicol (MIC 1.56 µg ml^{-1}) (Table 5). The MIC for trimethoprim and nitrofurantoin was 6.25 µg ml^{-1}. The sensitivity to furazolidone and neomycin was considered equivocal (both 12.5 µg ml^{-1}), and varying levels of resistance (based on likely achievable antibiotic concentrations in treated larvae) were assumed for ampicillin, amoxycillin, co-trimazine, sulphadiazine, sulphamethazine, streptomycin and trimethoprim/sulphamethazine (MIC for all >25 µg ml^{-1}).

Table 5. Results of *in vitro* antibiotic minimum inhibitory concentration assays for *Vibrio harveyi* isolated from *Jasus verreauxi* larvae during the original disease outbreak. S: sensitivity; E: equivocal; R: resistant

Antibiotic	Minimum inhibitory concentration (µg ml^{-1})	Result[a]
Oxolinic acid	0.2	S
Oxytetracycline	0.78	S
Chloramphenicol	1.56	S
Trimethoprim	6.25	S
Nitrofurantoin	6.25	S
Furazolidone	12.5	E
Neomycin	12.5	E
Ampicillin	>25	R
Amoxycillin	>25	R
Co-trimazine (1/5)	>25	R
Sulphadiazine	>25	R
Sulphamethazine	>25	R
Streptomycin	>25	R
Trimethoprim + Sulphamethazine (1/5)	>25	R

[a]Based on likely achievable antibiotic concentration in treated larvae

Histology

Apparently healthy phyllosomas had no bacteria evident in the lumen of hepatopancreas tubules (Figs. 1 & 3), gut or hemolymph. The hepatopancreas tubules of apparently healthy larvae were well organised and the hepatopancreocytes and connective tissue appeared normal (Fig. 3). The gut of luminous phyllosomas contained masses of bacteria, and foci of necrosis with evidence of hemocyte aggregation near necrotic areas (Figs. 2 & 4). Most of the hepatopancreas tubules of moribund larvae were atrophic with necrosis of hepatopancreatocytes, and most contained bacteria in the lumen (Figs. 5 & 6). The hemal space under the carapace of moribund larvae contained areas of eosinophillic hemolymph containing low numbers of bacteria (Fig. 5). Sloughing of necrotic hepatopancreatocytes into the lumen of hepatopancreas tubules was also noted (Fig. 7).

Experimental infections

Trial 1

Survival in Beaker A after 7 d was 40% and *Vibrio harveyi* was reisolated from all 3 of the larvae that died (Table 1). All of the phyllosomas in beaker C (10^7 *V. harveyi* ml^{-1} bath) had died by 3 d post exposure (PE) and *V. harveyi* was reisolated from all dead larvae. In contrast none of the phyllosomas in Beaker B (10^6 *V. harveyi* ml^{-1} bath) were luminous after 7 d; however, 1 phyllosoma was unaccounted for and may have been cannibalised (Table 1). Both fed and unfed control larvae had 100% survival after 7 d (Table 1).

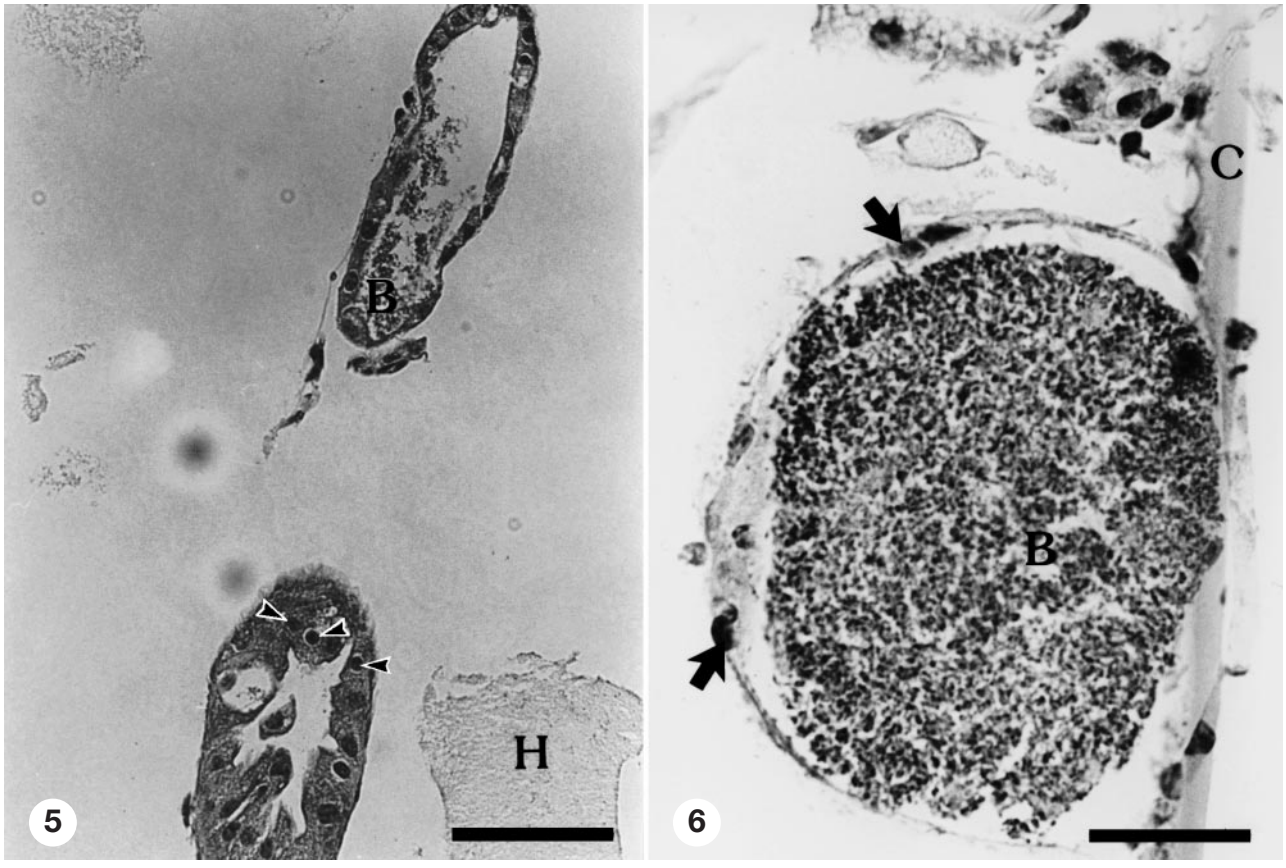
Trial 2

Mortalities were first recorded 2 d PE, when 2 dead and luminous phyllosomas were removed from Beaker D (Fig. 8). Mortalities were first observed in Beakers A and B at 3 d PE. When the experiment was terminated after 7 d PE, survival was 35% in the damaged larvae (pooled from Beakers A [40% survival] and B [30% survival]), and 50% in the undamaged larvae exposed to *Vibrio harveyi* in Beaker D. Survival of undamaged, unexposed phyllosomas in Beaker C was 100% (Fig. 8). Pure cultures of *V. harveyi* were reisolated from all of the phyllosomas that died during this experiment. When bacteriology was performed on the phyllosomas which survived 7 d, the only bacteria isolated were light growths of pure *V. harveyi* recovered only from 2 of the survivors in Beaker A, and from 1 of the survivors in Beaker D.

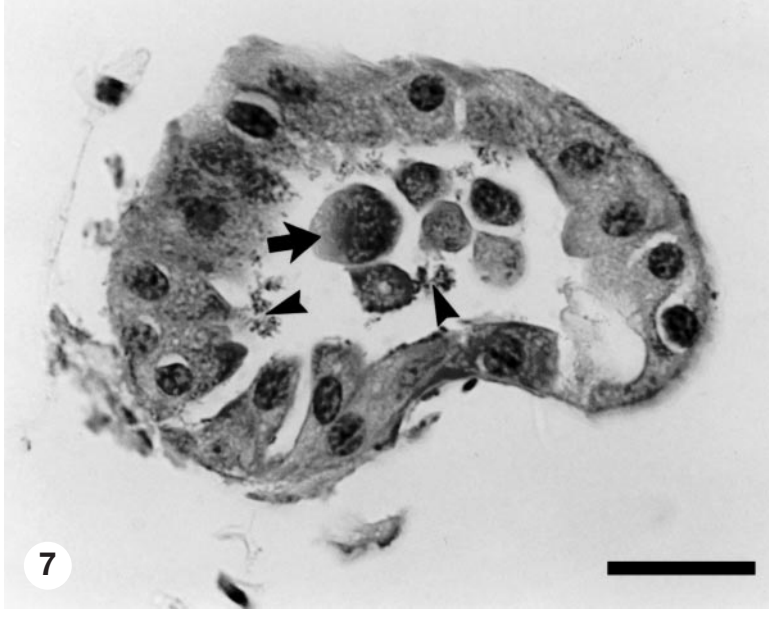
In vivo antibiotic trials

Trial 3

Two of the 5 control phyllosomas exposed to a bath of 5×10^6 *Vibrio harveyi* ml^{-1} had died by Day 7 PE (Table 2); however, no *V. harveyi* were reisolated from these individuals. The survival of phyllosomas exposed to the 50:10 mg l^{-1} combination of sulphamethazine and trimethoprim (Beaker D) and 100 mg l^{-1} oxytetracycline (Beaker F) was 80%. One phyllosoma died in Beaker D after 5 d PE and *V. harveyi* was reisolated from this individual. The phyllosoma which died in Beaker F was found dead after 7 d PE, and showed signs of deformity in a moult. *V. harveyi* was not reisolated from this individual. Only 1 of the phyllosomas exposed to 0.1 mg l^{-1} Nifurpirinol in Beaker E survived. All dead phyllosomas in Beaker E were luminous and *V. harveyi* was reisolated from all of these larvae. No



Figs. 5 & 6. *Jasus verreauxi.* Fig. 5. Two adjacent hepatopancreas tubules of a luminous phyllosoma. The lower tubule contains necrotic hepatopancreocytes with pyknotic nuclei (arrowheads) while the upper tubule is atrophic with necrotic hepatopancreocytes and numerous bacteria (B) in the lumen. Note also scattered bacteria in hemolymph (H). Scale bar = 65 µm. Fig. 6. Higher magnification of a hepatopancreas tubule of a luminous phyllosoma. Note complete atrophy and necrosis of hepatopancreocytes (arrows) and the lumen is packed with bacteria (B). C = cuticle. Scale bar = 25 µm



phyllosomas exhibited clinical signs of *V. harveyi* infection and survival was 100% in Beakers A (control, no bacteria) and C (20/100 mg l^{-1} combination of trimethoprim/sulphamethazine).

Trial 4

Mortalities were recorded after 48 h for the phyllosomas exposed to 10^4 *Vibrio harveyi* ml^{-1} at both 16 and 20°C, and after 7 d their survival rates at these temperatures were 10 and 30%, respectively

Fig. 7. *Jasus verreauxi.* A hepatopancreas tubule of a luminous phyllosoma. Note necrotic hepatopancreocytes (arrow) and bacteria (arrowheads) in the lumen. Scale bar = 25 µm

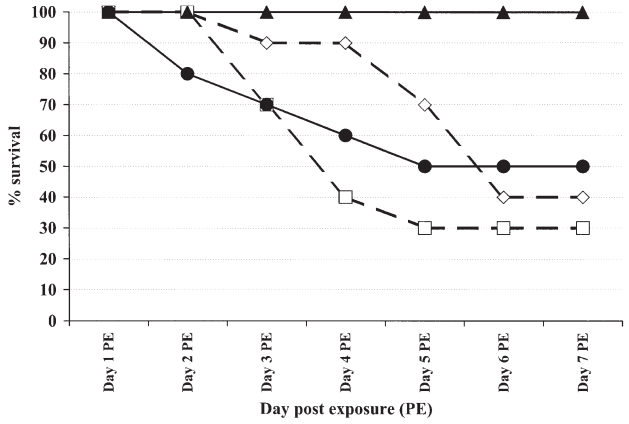


Fig. 8. Survival of damaged and undamaged phyllosoma larvae of *Jasus verreauxi* (4to to 5th instar) in Trial 2 at 22°C. (—◇—) Beaker A, injured, no *Vibrio harveyi* added; (—□—) Beaker B, injured, 10^4 CFU ml^{-1} *V. harveyi* added; (—▲—) Beaker C, not injured, no *V. harveyi* added; (—●—) Beaker D, not injured, 10^4 CFU ml^{-1} *V. harveyi* added

(Fig. 9). The survival rate of larvae exposed to 10^4 *V. harveyi* ml^{-1} was significantly lower ($p = 0.0002$) than the survival of larvae from all other treatments. Only 65 and 50% of untreated larvae had survived after 7 d at 16 and 20°C, respectively. Survival of larvae given 120 mg l^{-1} trimethoprim/sulphadimidine (1/5) + 10^4 *V. harveyi* ml^{-1} after 7 d at 16 and 20°C was 80 and 70%, respectively, and was not significantly different to survival of untreated larvae ($p > 0.05$). Only 2 mortalities were recorded in larvae exposed to 120 mg l^{-1} trimethoprim/sulphadimidine (1/5), giving a survival rate after 7 d of 95% at both 16 and 20°C (Fig. 9), which was significantly higher than that of untreated larvae ($p < 0.05$), but not significantly different ($p > 0.05$) from survival of larvae given 120 mg l^{-1} trimethoprim/sulphadimidine (1/5) + 10^4 *V. harveyi* ml^{-1}. Pure cultures of *V. harveyi* were recovered from all larvae that died during this experiment, and no bacteria were isolated from surviving larvae.

DISCUSSION

These outbreaks of disease in *Jasus verreauxi* phyllosoma larvae were associated with the presence of the luminous bacterium *Vibrio harveyi* and were the first records of luminous vibriosis in phyllosoma larvae of spiny lobsters. The original outbreak of luminous vibriosis followed an increase in water temperature from 19 to 23°C, which is consistent with *V. harveyi* acting as a pathogen of crustaceans at warmer water temperatures (>22°C). However, the second disease outbreak showed that luminous vibriosis could occur at 20°C without an increase in water temperature and further experiments reproduced the disease in the 4th to 5th instar phyllosomas at water temperatures as low as 16°C.

It has been established that epizootics of luminous vibriosis in penaeid shrimp larvae are associated with elevated numbers of luminous bacteria in growout ponds (Lavilla-Pitogo et al. 1998). Our experimental bath exposures with this *Vibrio harveyi* isolate demonstrated that it was possible to infect *Jasus verreauxi* phyllosomas via immersion in water containing $\geq 10^4$ *V. harveyi* ml^{-1} and produce mortality rates approximating those observed in the disease outbreaks. We also observed phyllosomas with clinical signs of luminous vibriosis being cannibalised by apparently healthy phyllosomas, which themselves subsequently became luminous and moribund. This suggests that *V. harveyi* infections can also be transmitted via infected food, a suggestion supported by histopathology indicating that the digestive tract is the main target organ.

Inadvertent injury to the phyllosomas is a variable that may account for some of the inconsistencies we observed with our attempts to experimentally infect phyllosomas. Our data indicated that injured phyllosomas were more susceptible to infection, and it is possible that some larvae were accidentally injured while being transferred into experimental beakers, thus increasing the chances of infection in some beakers. Once injured phyllosomas became infected, bacteria appeared to rapidly proliferate internally, and the phyllosomas became luminous. The luminous phyllosomas then appeared to provide a source of virulent bacteria which increased the likelihood of infecting the

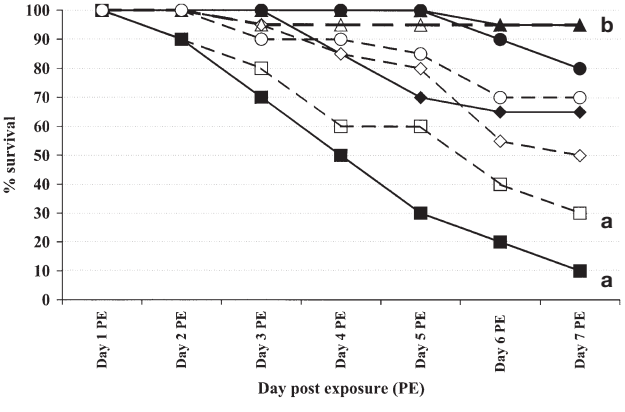


Fig. 9. Survival of phyllosoma larvae of *Jasus verreauxi* (4th to 5th instar) in Trial 4 during a *Vibrio harveyi* disease outbreak at 16°C (—▲—, —●—, —◆—, —■—) or 20°C (—△—, —○—, —◇—, —□—) when given either no treatment (◆ or ◇), 10^4 CFU ml^{-1} *V. harveyi* (■ or □), 120 mg l^{-1} sulphadimidine/trimethoprim (▲ or △), or 10^4 CFU ml^{-1} *V. harveyi* and 120 mg l^{-1} sulphadimidine/trimethoprim (● or ○). a: significantly lower survival than all other treatments, b: significantly higher survival than untreated larvae

remaining phyllosomas in the treatment (within the limited duration of the 7 d trial period) through cannibalism or decomposition. This may explain why mortalities were observed in Trial 1 at 10^4 *Vibrio harveyi* ml^{-1}, but not at 10^6 *V. harveyi* ml^{-1}.

The recovery of *Vibrio harveyi* from dead and surviving larvae in Beaker A in Trial 2, where no *V. harveyi* was added, suggests that the bacterium may have been introduced into these beakers with apparently healthy larvae either externally (as larvae were not surface sterilised before being transferred to experimental beakers) or internally. Bacteriology results from non-luminous, apparently healthy larvae examined during the original disease outbreak showed that these could still carry low level *V. harveyi* in their internal organs. Since it is not known how many of the apparently healthy larvae used in experimental infections may have been asymptomatic carriers of *V. harveyi* infections, the results of our experimental infections cannot be strictly regarded as fulfilling Koch's postulates. However, repeated isolation of pure cultures of *V. harveyi* from luminous larvae in 2 disease outbreaks examined 1 yr apart, and also from luminous larvae in our experimental infections, indicates that this bacterium is an important opportunistic pathogen of phyllosoma larvae of *Jasus verreauxi* under certain conditions, particularly when larvae are injured. Another inconsistency which occurred on more than one occasion was that *V. harveyi* was not reisolated from larvae that died exhibiting clinical signs of infection. This may have been due to ethanol contamination during inoculation of culture plates.

The results of the experiments conducted in the present study indicated that antibiotics could be used to significantly improve the survival of larvae during outbreaks of luminous vibriosis in phyllosoma cultures. Our MIC data suggest the efficacy of the trimethoprim/sulphamethazine (1/5) and trimethoprim/sulphadimidine (1/5) treatments at 120 mg l^{-1} was probably due to the 20 mg l^{-1} of trimethoprim, which was over 3 times the MIC for this antibiotic. Furthermore, oxytetracycline (MIC 0.78 mg l^{-1}) may have provided protection at much lower doses than the 100 mg l^{-1} used here, which might avoid the problems observed with deformities of larvae. However, Baticados et al. (1990) suggested that problems with antimicrobial drug-induced deformities in larval *Penaeus monodon*, together with the potential for development of antibiotic resistant strains of *Vibrio harveyi* (see Baticados et al. 1990, Karunasagar et al. 1994, Pillai & Jayabalan 1996) were the main reasons why prophylactic use of antimicrobials should be discouraged in penaeid shrimp culture. We consider that these problems associated with antibiotic use are just as relevant for larviculture of spiny lobsters as they are for penaeid shrimp culture.

It is recommended, therefore, that modifying husbandry practices to avoid outbreaks of luminous vibriosis should be the primary method used to combat this disease in *Jasus verreauxi* phyllosomas. Different management procedures could include minimising mechanical damage to larvae, controlling the bacterial flora of the culture water through introducing microalgae (Igarashi et al. 1990), use of probiotics (Rengpipat et al. 1998) or immunostimulants (Scholz et al. 1999), and controlling bacteria in live food by using methods such as probiotics, or surface sterilisation (Munro et al. 1999).

Acknowledgements. We thank Toni Wagner for microbiology support. This project was conducted as part of an augmenting PGSF-funded programme CO1606. J. Booth and 2 anonymous referees provided valuable comments on drafts of the manuscript.

LITERATURE CITED

Anhalt JP, Washington JA (1991) Preparation and storage of antimicrobial solutions. In: Balows A (ed) Manual of clinical microbiology, 5th edn. American Society for Microbiology, Washington DC, p 1199–1200

Austin B, Lee JV (1992) Aeromonadaceae and Vibrionaceae. In: Board RG, Jones D, Skinner FA (eds) Identification methods in applied and environmental microbiology. Society for Applied Bacteriology, Oxford, p 163–182

Baticados MCL, Lavilla-Pitogo CR, Cruz-Lacierda ER, de la Pena LD, Sunaz NA (1990) Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. Dis Aquat Org 9:133–139

Baumann P, Baumann L, Mandel M (1971) Taxonomy of marine bacteria: the genus *Beneckea*. J Bacteriol 107: 268–294

Beuchat LR (1974) Combined effects of water activity, solute, and temperature on the growth of *Vibrio parahaemolyticus*. Appl Microbiol 27:1075–1080

Booth JD, Kittaka J (1994) Growout of juvenile spiny lobster. In: Phillips BF, Cobb JS, Kittaka J (eds) Spiny lobster management. Blackwell Scientific Publications, Oxford, p 424–445

Bowser PR, Rosemark R (1981) Mortalities of cultured lobsters, *Homarus*, associated with a moult death syndrome. Aquaculture 23:11–18

Bryant TN, Smith KR (1991) Bacterial identifier, Version M 1.0. Blackwells, Oxford

Bryant TN, Lee JV, West PA, Colwell RR (1986) A probability matrix for the identification of species of *Vibrio* and related genera. J Appl Bacteriol 61:469–480

Carson J, Statham P (1993) The inhibition by ionophores *in-vitro* of an enterococcal-like pathogen of rainbow trout, *Oncorhynchus mykiss*. Vet Microbiol 36:253–259

Cawthorne RJ, Lynn DH, Despres B, MacMillan R, Maloney R, Loughlin M, Bayer R (1996) Description of *Anophryoides haemophila* n. sp. (Scuticociliatida: Orchitophryidae), a pathogen of American lobsters *Homarus americanus*. Dis Aquat Org 24:143–148

Evans LH, Brock JA (1994) Diseases of spiny lobsters. In: Phillips BF, Cobb JS, Kittaka J (eds) Spiny lobster management. Blackwell Scientific Publications, Oxford, p 461–472

Fisher WS, Nilson EH, Shleser RA (1975) Effect of the fungus *Haliphthoros milfordensis* on the juvenile stages of the American lobster *Homarus americanus*. J Invertebr Pathol 26:41–45

Fisher WS, Nilson EH, Steenbergen JF, Lightner DV (1978) Microbial diseases of cultured lobsters: a review. Aquaculture 14:115–140

Furniss AL, Lee JV, Donovan TJ (1978) The vibrios. Monograph Series 11, Public Health Laboratory Service. Her Majesty's Stationery Office, London

Hooker SM, Jeffs AG, Creese RG, Sivaguru K (1997) Growth of captive *Jasus edwardsii* (Hutton) (Crustacea: Palinuridae) in north eastern New Zealand. Mar Freshw Res 48:903–909

Igarashi MZ, Kittaka J, Kawahara E (1990) Phyllosoma culture with inoculation of marine bacteria. Nippon Suisan Gakkaishi 56:1781–1786

Illingworth J, Tong LJ, Moss GA, Pickering TD (1997) Upwelling tank for culturing rock lobster (*Jasus edwardsii*) phyllosomas. Mar Freshw Res 48:911–914

Karunasagar I, Pai R, Malathi GR, Karunasagar I (1994) Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. Aquaculture 128:203–209

Kittaka J (1994) Larval rearing. In: Phillips BF, Cobb JS, Kittaka J (eds) Spiny lobster management. Blackwell Scientific Publications, Oxford, p 403–423

Kittaka J (1997) Application of ecosystem culture method for complete development of phyllosomas of spiny lobster. Aquaculture 155:319–331

Kittaka J, Ono K, Booth JD (1997) Complete development of the green lobster, *Jasus verreauxi* from egg to juvenile. Bull Mar Sci 61:57–71

Lavilla-Pitogo CR, Leano EM, Paner MG (1998) Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent vibrios in the rearing environment. Aquaculture 164:337–349

Moss GA, Tong LJ, Illingworth J (1999) Effects of light levels and food density on the growth and survival of early stage phyllosoma larvae of the rock lobster *Jasus edwardsii*. Mar Freshw Res 50:129–134

Munro PD, Henderson RJ, Barbour A, Birbeck TH (1999) Partial decontamination of rotifers with ultraviolet radiation: the effect of changes in the bacterial load and flora of rotifers on mortalities in start feeding turbot. Aquaculture 170:229–244

Pillai D, Jayabalan N (1996) Sensitivity of luminous bacteria *Vibrio harveyi* to nine selected antibiotics. Indian J Fish 43:399–402

Rengpipat S, Phianphak W, Piyatiratitivorakul S, Menasveta P (1998) Effects of probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167:301–313

Scholz U, Garcia Diaz G, Ricque D, Cruz Suarez LE, Vargas Albores F, Latchford J (1999) Enhancement of vibriosis resistance in juvenile *Penaeus vannamei* by supplementation of diets with different yeast products. Aquaculture 176:271–283

Sindermann CJ (1990) Principal diseases of marine fish and shellfish, Vol 2. Diseases of marine shellfish, 2nd edn. Academic Press, New York, p 93–144

Stewart JE (1980) Diseases. In: Cobb JS, Phillips BF (eds) The biology and management of lobsters, Vol 1. Academic Press, New York, p 301–342

Stewart JE (1984) Lobster diseases. Helgol Meeresunters 37: 243–254

Unestam T (1973) Fungal diseases of Crustacea. Rev Med Vet Mycol 8:1–20

West PA, Colwell RR (1984) Identification and classification of Vibrionaceae—an overview. In: Colwell RR (ed) Vibrios in the environment. John Wiley & Sons, New York, p 285–363

Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: January 17, 2000; Accepted: September 7, 2000
Proofs received from author(s): October 25, 2000