Improved Early Survival of Molluscs: Sydney Rock Oyster (*Saccostrea glomerata)*

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1.0 SUMMARY

Continuation of an otherwise very successful 10 year breeding program to produce fast growing disease resistant Sydney rock oysters *Saccostrea glomerata* depends on whether diseases of larval and juvenile *S. glomerata* below about 2 mm can be overcome. The objective of this project has been to systematically address and overcome both of these diseases. Five complimentary strategies have been adopted to investigate mass mortality diseases of larval and juvenile *S glomerata.*

1. Appraisal of all existing epidemiological information including in-house hatchery records and reports of previous epizootics and monitoring and documentation of ongoing hatchery and nursery operations at the PSFC.
2. Collection and preservation of larvae and spat for histopathological examination and possible diagnosis in of infective agents linked to disease episodes.
3. Experimental investigations of:
    - possible causes
    - predisposing and ameliorating factors to disease outbreaks
    - the effectiveness of alternative methods of disease control and prevention.
4. Determination and adoption of optimal physio-chemical rearing conditions for *S. glomerata* larvae.
5. Use of probiotic agents to provide more favourable and stable micro-flora environments within hatchery rearing system - (this strategy is now being addressed separately in a collaborative project initiated in September 1999).

1.1 Results - Larval Disease

1.1.1 Epidemiological investigations

The following important information on the clinical manifestation of the larval mortality syndrome was gained from analyses of hatchery records for the period 1990 to 2000.

1. There has been a continuing trend of improving hatchery performance with *S. glomerata* over the past decade beyond that achieved at the PSFC up to 1990.
2. First non specific clinical signs of the disease, anorexia, occurs most commonly (about 75% of the time) on days 3 to 5 but also occurs as early as day 2 and as late as day 8.
3. Sub-lethal effects on growth rate of diseased larvae coincident with anorexia are evident from about day 4.
4. The larval disease is characterised by elevated mortality culminating in complete mortality on average by day 14.
5. Whether or not the disease occurs and what proportion of larvae survive to settlement stage are not related to apparent egg quality as indicated by yield of D veligers from eggs. Like-wise, survival rate of D veliger larvae is not correlated to subsequent yield of spat.

Useful epidemiological information gained from the same analyses of hatchery records was as follows:

1. There is a marked seasonal variation in the incidence of mass larval mortality. Indeed the calendar year can be divided into halves of contrasting hatchery fortunes for *S. glomerata.* The first half of the year (January to June), is characterised by chronically poor survival with successful hatchery runs being achieved only once in every two to six attempts i.e. a success rate of 17 to 40%. By contrast chances of success during the second half of year (July to December), are much improved, ranging from 40 to 100%.

2. Dry-out and disinfection of the hatchery markedly reduced the occurrence of larval disease. Following a minimum hatchery disinfection and dry-out period of four weeks, the probability of a successful hatchery operation decreases over successive runs from about 60% on the first run to 40% on the second and 25% on the third. These findings highlighted the critical need of regular programmed dry-out and disinfection of hatcheries intending to produce *S. glomerata* and the desirability of a modular design for any such hatchery.
3. Use of commercial scale 20000L larval rearing tanks substantially improves chances of success.
4. The mass mortality disease appears virulent only to larvae of *S.glomerata* and a related native rock oyster, *S. cuculata*. Many other bivalves including two species of clam, two species of scallop, two species of pearl oysters, blue mussels and the Australian flat oyster and the pacific oyster, are regularly produced in multi-million batches at the PSFC hatchery without significant problems. This is true even when the larvae of such species are hatchery reared in company with afflicted batches of *S. glomerata* larvae.

1.1.2 Pathology

Numerous samples of symptomatic larvae have been submitted to standard light microscope histological examination by specialist pathologists including Dr Judith Handlinger (Mt Pleasant Laboratories, Launceston), Dick Callinan (Wollongba Veterinary Laboratory, NSW, DPI) and Dr Ralph Elston (USA). Many samples of larvae and rearing water have also been submitted to Dr Peter Hanna and colleagues (Deakin University, Vic) for bacterial examination using monoclonal antibodies specific for particular species of *Vibrio* bacteria and to Dr Jeremy Carsons (Mt Pleasant Laboratories, TASDPIF, Launceston). Samples have been submitted for electron microscopy (TEM and SEM) examination to Dr Mike Hine (NIWA- New Zealand) and to Dr Alex Hyatt (AAHL/(CSIRO), Geelong,Vic.). Four lots frozen larvae have similarly been air-freighted to La Tremblade, France for specialist examination by Dr Tristan Renault (IFREMER) using PCR probes recently developed for detection of a herpes virus that causes a very similar anorexia disease of hatchery reared pacific oyster larvae and spat in western Europe.

1.1.3 Experimental investigations

Results of an experiment in December 1997 showed that disease was transferred when larvae were reared in a separate facility but in contact with 1.0 micron filtered seawater previously exposed to diseased larvae. Histopathological and microbiological evidence supported the conclusion that the disease is probably bacterial in origin and probably associated with bacteria that can persist for long periods between successive production cycles on wet surfaces within the bivalve hatchery or associated seawater storage and plumbing systems.

Preliminary research to evaluate probiotics in combating larval disease was conducted in collaboration with the Department of Cell Biology, UTS. Several strains of potentially pathogenic bacteria were isolated from surfaces of vessels containing diseased larvae and identified as either *Vibrio* or *Aeromonas* species. All but one strain were found sensitive to tetracycline and all were sensitive to strains of probiotic bacteria. Results of a trial to test prophylactic use of probiotic bacteria on *S. glomerata* larvae were very encouraging and have served as the basis of a collaborative 3 year SPIRT funded project.

1.1.4 Optimisation of rearing conditions

Results of the first of three experiments showed that sperm should be stored at or below 15^0C and preferably used within an hour of collection. In the second experiment, storage periods over which eggs retained high rates (>90%) of fertilisation were found to decrease from 2h at 15^0C to less than 30 mins at 30^0C. Results of the third experiment showed optimum larval rearing temperature to be about 29^0C which is 5^0C higher than a temperature of 24^0C routinely used to rear *S glomerata* larvae at the PSFC.

1.1.5 Experimental use of probiotics

In December 1999, a joint 3 year R&D project was initiated by UTS and NSW Fisheries to further evaluate use of probiotics for combating mass mortality diseases of larval and early juvenile *S. glomerata*. Although only one experiment has been conducted at the time of this final report, results showed that sterilization of sea water followed by re inoculation with one or a combination of 3 different selected species of "friendly" bacteria raised survival of 8 day old *S, glomerata* larvae from a base level of only 9% (typical of disease episodes) to a range of 45 to 68%. The latter survival rates are among the highest achieved with *S. glomerata* larvae. Benefits conferred by sterilization and re-inoculation of seawater with " friendly" bacteria were equally dramatic in relation to enhanced larval growth.

1.2 Results- Juvenile (Spat) Disease

1.2.1 Epidemiological investigations

A review of hatchery records for the period 1988 to showed that first manifestation of the disease has continued to range widely (from 7 to 43 days after settlement) and that the disease is confined to spat below 2 mm shell-height. In contrast to the larval disease, no seasonal trends were apparent in the relative occurrence of mass mortality of juvenile *S. glomerata*.

1.2.2 Pathology

As with the larval disease, numerous samples of diseased spat have been submitted to the same array of specialist pathologists using a similar array of histopathological techniques. All resultant pathology reports contained all or some of the following elements.

1. Histopathological examination revealed a range of conditions from asymptomatic to severely diseased individuals among mass mortality spat.
2. Lesions seen in all obviously diseased individuals comprised some or all of the following
    - mild to severe focal to diffuse inflammation or necrosis of connective tissue sometimes associated with rod shaped bacteria
    - diffuse mild to severe degeneration of retractor muscles associated with infiltration by large numbers of haemocytes.
    - parietal mantle surface often severely effected with exudation of proteinacious fluid and cell debris.
    - partial dissolution of the hinge ligament associated with moderate to many bacteria of various morphological forms including filamentous.

Although no other potential causative agents were seen associated with the above lesions, similar but less common and less severe lesions to the above were often seen among the batches of

apparently healthy spat. High numbers of bacteria have often been detected on external surfaces of spat reared at high densities of 100 000 to 200 000 per 450 mm diameter nursery screens. However reports of invasive bacterial infections have been confined to dead or moribund gaping spat. In this sense the conclusion of Dick Callinan in 1990 that histopathology was more indicative of proteolytic bacterio-toxins than of any other potential agent capable of inducing this type of histopathology, must still be regarded as the most plausible albeit unproven hypothesis put forward to date. The only exception to the above was a major mass mortality event in 1999 that was ascribed by each of four pathologists consulted to an aggressive and invasive ciliate (*Euronema sp*.) infection. Re-examination of archived samples of diseased spat however failed to detect evidence of involvement of these ciliates in other disease episodes.

1.2.3 Experimental investigations

An experiment was run to test hypotheses that:

- the primary cause of the disease is the development of large concentrations of virulent strains of bacteria and exotoxins thereof associated with the use of ground scallop shell for producing single seed spat.
- manifestation of mass mortalities (as opposed to sub-clinical disease in apparently healthy stock) depends on a triggering factor especially trauma or other stressors associated with grading and counting of small spat.
- mass mortalities, if caused directly or indirectly by bacteria, could be prevented or mitigated against with prophylactic use of antibiotics.

Absence of the disease from all eight experimental treatments precluded definitive findings on the cause(s), control or prevention of the disease. Nevertheless an important inadvertent finding was that cultchless settlement using epinephrine induction of metamorphosis and omission of periodic grading of small *S.glomerata* spat, drastically reduces hatchery labour requirements without adversely effecting the rate or uniformity of growth.

2.0 BACKGROUND AND JUSTIFICATION

The 120 year old Sydney Rock Oyster industry employs more people (about 1200) than any other form of aquaculture in Australia. However, it has suffered a 40% decline from peak production levels of about 130 000 bags per annum maintained during the 1970's, representing lost gross annual revenue of about $20 million and hundreds of jobs in regional NSW from Tweed Heads to Eden. Also as a consequence of this decline in production, domestic market share has been lost to the faster growing pacific oyster produced predominantly from single-seed hatchery stock in Tasmania, South Australia and New Zealand. Significant contributing factors to the decline include:

- Escalating costs associated with inherently slow growth and hence protracted turn-off time (3 to 4 years) of the native Sydney rock oyster (*Saccostrea glomerata).*
- High susceptibility of *S. glomerata* to fatal diseases, especially Winter Mortality and QX disease caused by intracellular parasites.

Over the past 10 years the Federal Government, through the Fishing Industry Research and Development Corporation (FRDC) grants and the NSW Government, has invested in excess of 1.0 million dollars in research to develop genetically improved *S. glomerata*. Third generation selected lines have been shown to confer significantly superior weight for age (in the order of 20%) over control non-selected lines (Nell, et al., 1999). Major advantages have also been demonstrated in relation to hatchery produced triploid Sydney rock oysters over diploid siblings. These advantages which have been shown to hold over a representative range of sites and farming techniques, include significantly faster weight for age (in the order of 30 to 40%), much more protracted retention of market condition and considerably enhanced resistance to disease especially winter mortality (Hand et al., 1998). Recent revision of this R& D includes an evaluation of whether the advantages conferred by triploidy can be combined and enhanced by using the progeny of selected line breeding stock. The selective breeding program has also been extended and expanded to target single and dual resistance to two important intracellular parasite diseases - Winter Mortality (haplosporidiasis) and QX disease (marteiliasis). The latter commonly causes annual mortalities above 10% on oyster farms in Northern NSW and on occasions such as oyster farms on the lower Georges River since 1995, financially ruinous losses above 80%.

Increased productivity using improved inter-tidal and deep-water suspended culture in conjunction with stocks genetically selected for rapid growth and disease resistance thus offers the best prospects for arresting the decline of the industry. Use of hatchery produced rather than wild caught *S. glomerata* spat is therefore becoming increasingly important to the NSW oyster industry. Hence it is essential that reliable hatchery production of the *S. glomerata* single spat is developed.

The successful transfer (widespread commercial adoption) of this technology is however subject to successful demonstration of reliable cost effective hatchery and nursery production technology for supply of juveniles (seed) to the industry. NSW has an absence of private sector mollusc hatcheries but also has an indefinite prohibition on the importation on seed oysters from existing interstate hatcheries. Under these circumstances NSW Fisheries has been attempting to supply commercial quantities of genetically improved seed oysters to the ailing industry out of its research hatchery at the Port Stephens Fisheries Centre (PSFC) since 1996. Lack of a private sector bivalve hatcheries has been due to insufficient demand for hatchery spat, due in turn to high cost compared with that of wild caught spat (<Aus$0.001ea.). High costs of hatchery produced spat can be ascribed to very high costs of establishing (=Aus$1 million) and operating (=$0.3million pa) conventional batch production oyster hatcheries.

Thus a supply and demand impasse (“*a Catch 22”*) exists for hatchery production of single seed *S. glomerata* spat. To break this impasse, demand at current market prices of $15 to $20 per thousand for 6 mm spat will need to reach or exceed an estimated 25 million per annum. This minimum production level would generate sufficient gross revenue ($375 000 to $500 000) to cover all costs and yield adequate internal rates of return (>15%pa) to justify the level of risk attached to this type of investment. While real demand does appear to be growing for genetically improved hatchery produced single seed oysters, major difficulties have been experienced over the past decade in routine hatchery and nursery rearing of *S. glomerata* at the PSFC.

Foremost among these problems is an intermittent disease that has often caused mass (60 to 90%) mortalities of small (<2 mm) Sydney Rock oyster spat since 1987 (Goard, 1990 and Nell et al., 1991). Exacerbating this problem are chronically low hatchery yields further compounded by intermittent catastrophic mortality, especially since 1996, of the larvae of *S. glomerata* and very occasional and far less severe disease outbreaks in several other bivalves for which aquaculture technology has also been developed at the PSFC over recent years. The latter include the Tasmanian scallop, *Pecten fumatus* (Heasman et al., 1998) and the clams, *Katylesia rhytiphora* (Nell et al., 1995) and *Tapes dorsatus,* (Patterson and Nell, 1997).

3.0 OBJECTIVES OF THE RESEARCH PROJECT

The objectives of this project were to improve the cost and reliability of hatchery producing *S. glomerata* by combating mass mortality diseases of both larvae and early juveniles.

4.0 OVERALL STRATEGY

Five complimentary strategies were adopted to investigate mass mortality diseases of both larva and juvenile *S glomerata.*

1. Appraisal of all existing epidemiological information including in-house hatchery records and reports of previous epizootics and monitoring and documentation of ongoing hatchery and nursery operations at the PSFC.
2. Collection and preservation of larvae and spat for histopathological examination and possible diagnosis of infective agents linked to disease episodes.
3. Experimental investigations of
    - possible causes
    - predisposing and ameliorating factors to disease outbreaks
    - the effectiveness of alternative methods of disease control and prevention.
4. Determination and adoption of optimal physio-chemical rearing conditions for *S. glomerata* larvae and juveniles.
5. Use of probiotic agents to provide more favourable and stable micro-flora environments within hatchery and nursery rearing systems. (This strategy is now being addressed separately in a collaborative project started in September 1999.)

5.0 INVESTIGATION OF MASS MORTALITY SYNDROME OF LARVAL S. GLOMERATA

5.1 Epidemiological Investigations

5.1.1 Methods

All available oyster hatchery performance information was extracted from diary records dating from September 1985. Reports compiled by Goard and Nell (1990), Nell et al. (1991) and by Frankish et al., 1991 were also reviewed in the light of more recent experience and information gathered. A search of published literature was also conducted in an attempt to uncover disease case-histories and research findings bearing close apparent resemblance to those afflicting larvae of *S. glomerata*.

5.2 Results and Conclusions

5.2.1 Re-evaluation of hatchery records and reports for the period 1985 to 1989

Frankish et al., 1991 in their report, *The Development of Hatchery Rearing of the Sydney Rock Oyster*, drew particular attention to the link between remarkable improvements in larval rearing success achieved from the mid 1970's. Improved forms of husbandry introduced over this period included:

- Use of coastal rather than estuarine water and its pre-settlement (6-7 days) and filtration (to 1μm nominal).
- Reduction in stocking rates from 10 to 15 eggs /ml down to 3 to 5 eggs/ml. (Fig 1)
- Progressive refinement of larval nutrition, including the species composition, microbial quality and feeding levels of micro-algae diets. Feeding levels originally set at 100 000 micro-algae cells/ml/day on the basis of overseas information are now adjusted daily to closely match demand (consumption rate). This involves a progressive increase from 7500 to 32000 cells/ml/day over the full duration of the larval rearing cycle.
- Use of much larger (20 000L) commercial scale rearing tanks providing far lower surface area to volume ratios and more stable physio-chemical conditions.

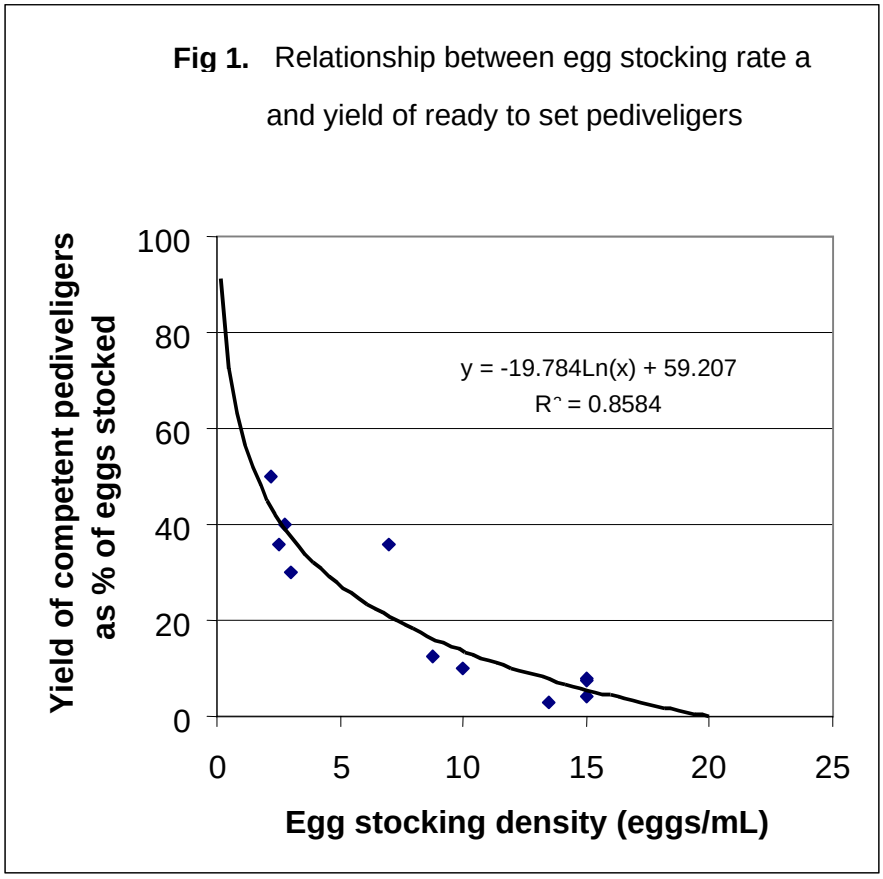
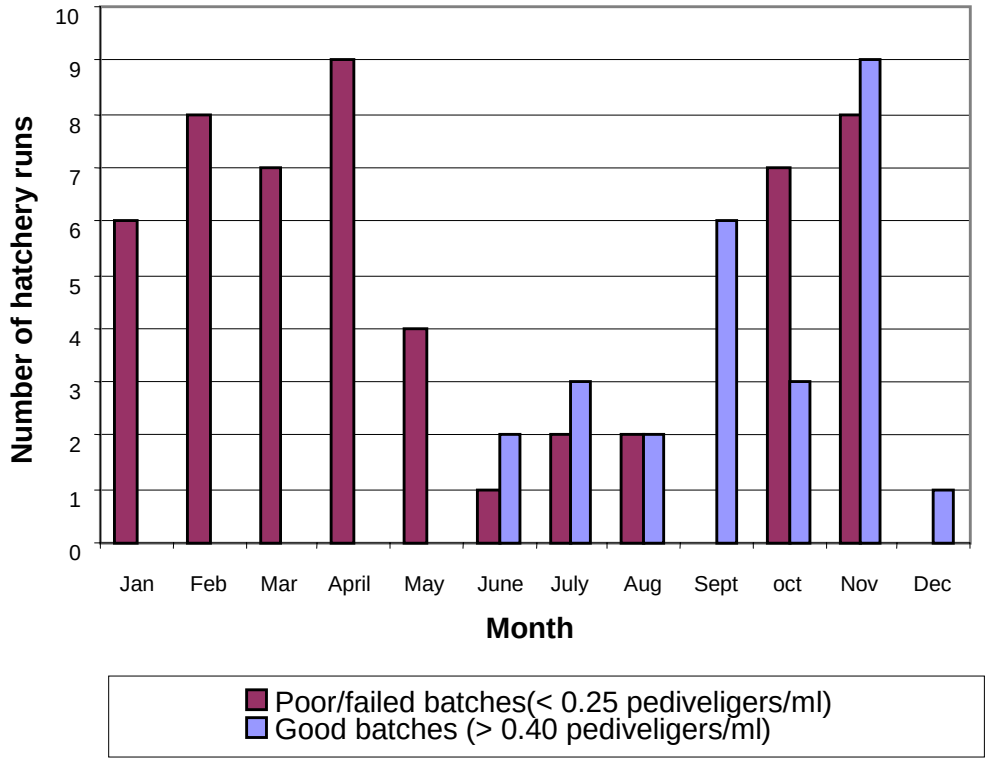


Fig 1. Relationship between egg stocking rate a and yield of ready to set pediveligers

Another important observation made by Frankish et al. (1991), from their review of hatchery records for the period 1985 to 1989, was a marked seasonal variation in hatchery success (Fig 2). While hatchery operations conducted during the first half of the calender year (January to June) almost invariably produced poor results (yields of ≤ 0.25 competent pediveligers/ml), those conducted during the second half of the year (July to December) often produced good results (yields of ≥ 0.40 competent pediveligers/ml).

Fig 2. Seasonal variation in hatchery success (as indicated by pediveliger yield/ml) in batches of *S. glomerata* 1985-1989 (from Frankish et al., 1991)



5.2.2 Analyses of hatchery records and reports for the period 1990-2000

Summarized hatchery performance data for the period 1985 to 1989 are compared with that of the subsequent period of 1990 to 2000 in Appendix 1. These data relate to a total of 70 hatchery runs.

Some salient information on the clinical manifestation of the larval mortality syndrome derived from these data are as follows:

1. A continuing trend of improving hatchery performance with *S. glomerata* over the past decade beyond that achieved by Frankish et al. 1991 is evident from a plot of successful and failed hatchery runs depicted in Fig. 3.

2. First non specific clinical signs of the disease, anorexia (Fig 4) occurred most commonly (about 75% of the time) on days 3, 4 or 5 but also occurred as early as day 2 and as late as day 8.

3. Sub-lethal effects on growth rate of diseased larvae coincident with anorexia were evident from about day 3 or 4 on (Fig 5).

4. The larval disease is characterised by elevated rates of mortality (Fig 6) culminating in complete mortality on average by day 14.

5. As indicated in Fig 7, whether or not the disease occurred and what proportion of larvae survived to settlement stage were not related to apparent egg quality as indicated by yield of D veligers from eggs. Like-wise survival rate of D veliger larvae was not strongly correlated to subsequent yield of spat.(Fig 8)

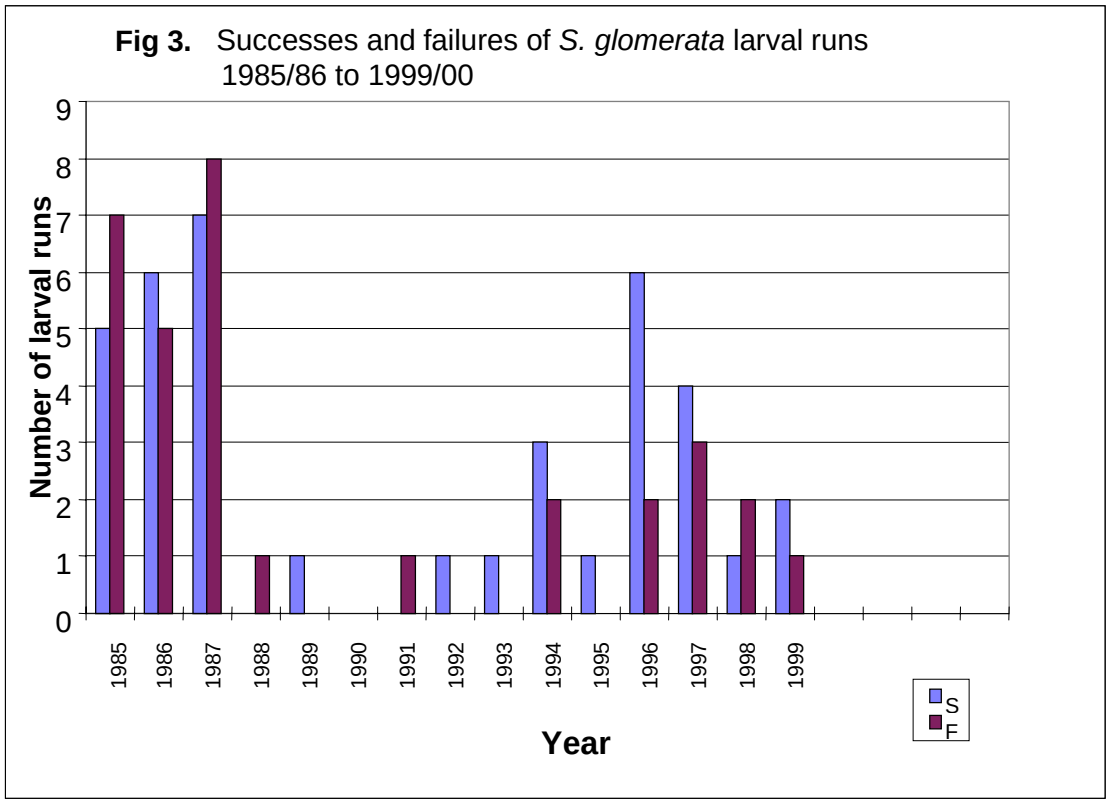


Fig 3. Successes and failures of *S. glomerata* larval runs 1985/86 to 1999/00

Fig 4. Day of manifestation of anorexia over the period 1985 to 1998 (n=51)

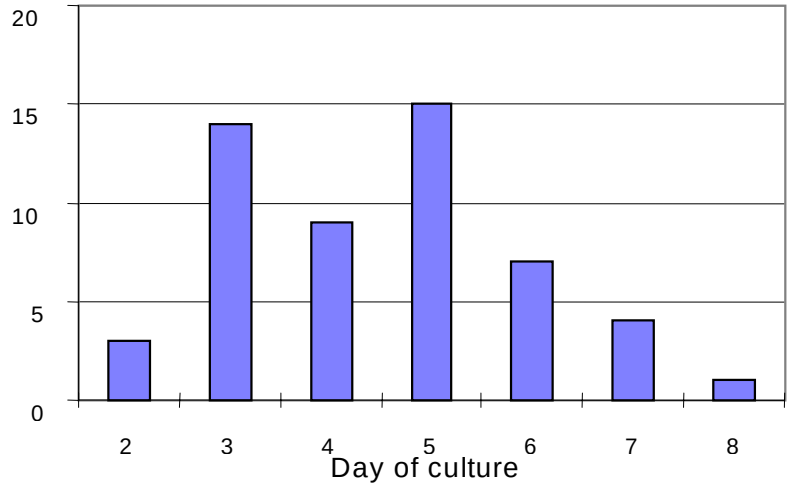
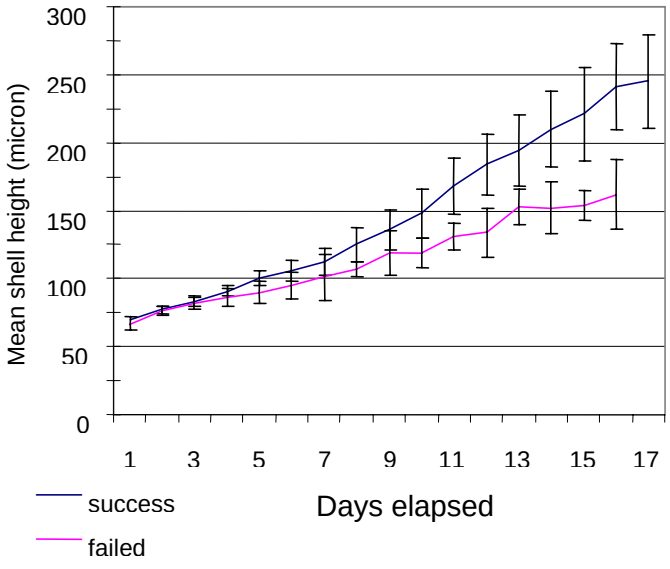


Fig 5. Mean +/- s.d. growth for successful (n=14) and failed (n=11) *S.glomerata* larval runs



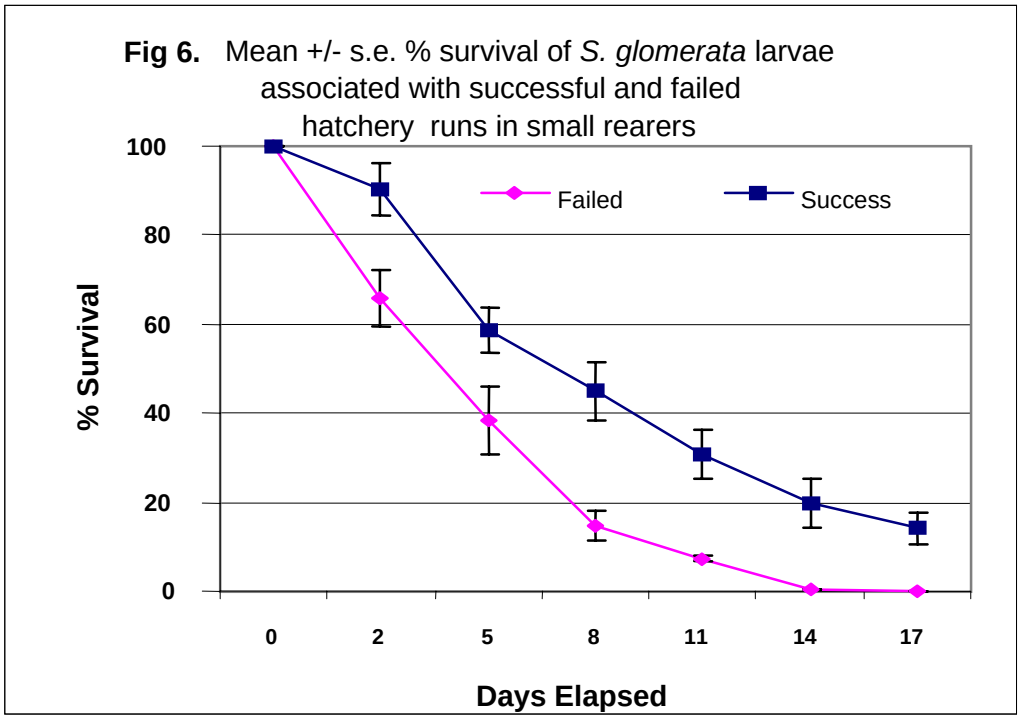


Fig 6. Mean +/- s.e. % survival of *S. glomerata* larvae associated with successful and failed hatchery runs in small rearers

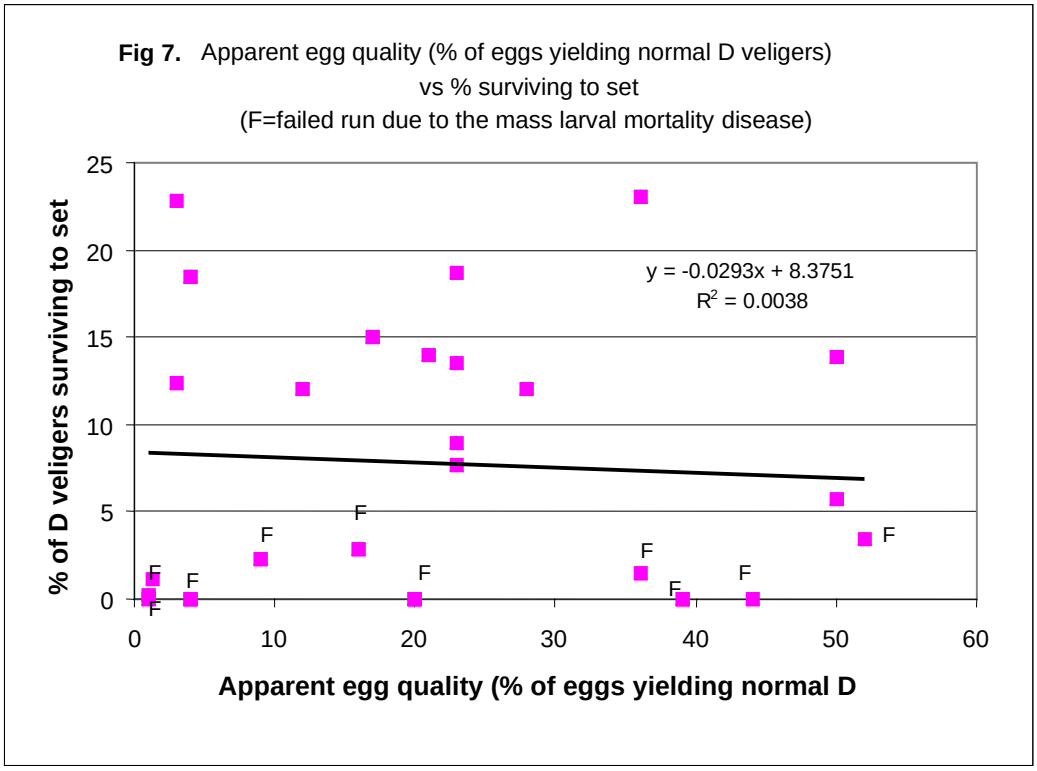


Fig 7. Apparent egg quality (% of eggs yielding normal D veligers) vs % surviving to set (F=failed run due to the mass larval mortality disease)

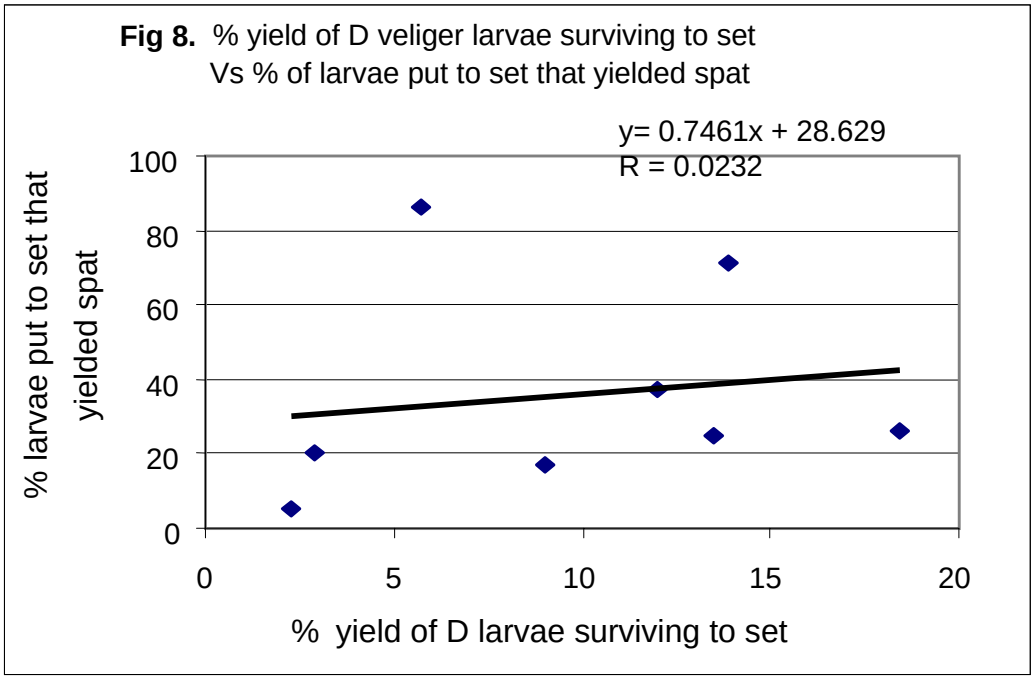


Fig 8. % yield of D veliger larvae surviving to set Vs % of larvae put to set that yielded spat

Some very useful additional information on the epidemiology of the disease was also derived from analysis of these 15 years of hatchery records.

1. The marked seasonal effect on the incidence of mass larval mortality syndrome recorded by Frankish et al., 1991(Fig 2) has continued over the past decade (Fig 9). As over the previous decade, the calendar year can be divided into halves of contrasting hatchery fortunes for *S. glomerata,* viz.:

- The first half of the year (January to June) based on records of 37 attempted hatchery runs, is characterised by chronically poor survival with successful hatchery runs only being achieved once in every two to six attempts i.e. a success rate each month ranging from 17 to 40%
- During the second half of year (July to December) chances of success, based on records of 34 attempted hatchery runs, are much improved ranging from 40 to 100%.
- Dry-out and disinfection of the new bivalve hatchery had a marked positive influence on the relative incidence of the larval disease of *S. glomerata*. Following a minimum hatchery disinfection and dry-out period of 4 weeks, the probability of a successful hatchery operations decreased over successive runs (Fig 10) from about 60% on the first run to 40% on the second and 25% on the third. By contrast the time elapsed from the previous hatchery run did not effect the incidence or severity of the disease (Fig 11). These findings highlight the critical importance of regular programmed dry-out and disinfection of hatcheries intending to produce *S. glomerata* and the desirability of a modular design for any future commercial bivalve hatchery in NSW.

2 As indicated in Fig 12, use of 20 000L tanks rather than 1 000L tanks within the new hatchery has increased the probability of successful hatchery runs. Of successful hatchery operations conducted in the new hatchery for which comprehensive survival data are available, 4 were conducted in small (1000L) experimental tanks and 10 in commercial scale (20 000L) rearing tanks. Within this data set, survival rates from first feeding D veliger stage to ready-to-set pediveliger stage was higher in 20 000L rearing tanks (mean ± s.d., 15.7± 4.8%; range 9 – 23%) than in the 1000L tanks (11.7± 3.5%; range 8 to 14%). However the reverse trend was

true for post-settlement survival that was higher for 1 000L tank reared larvae (46.3± 38.6%; range 5 to 86%) than for the 20 000L tank reared larvae (29± 7%; range 20 to 37%). Thus larger 20 000 L tanks have substantially improved the proportion of successful runs but within successful runs they have not provided a higher net yield of spat from D veligers (average yield 4.6%) than the smaller 1000L tanks (average yield 5.4%).

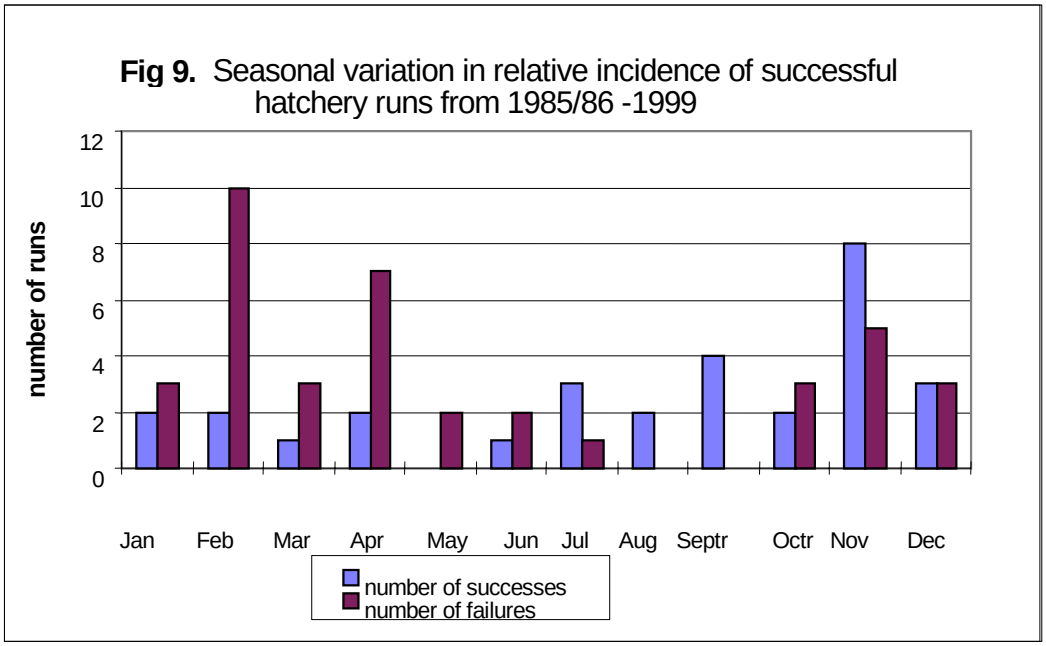


Fig 9. Seasonal variation in relative incidence of successful hatchery runs from 1985/86 -1999

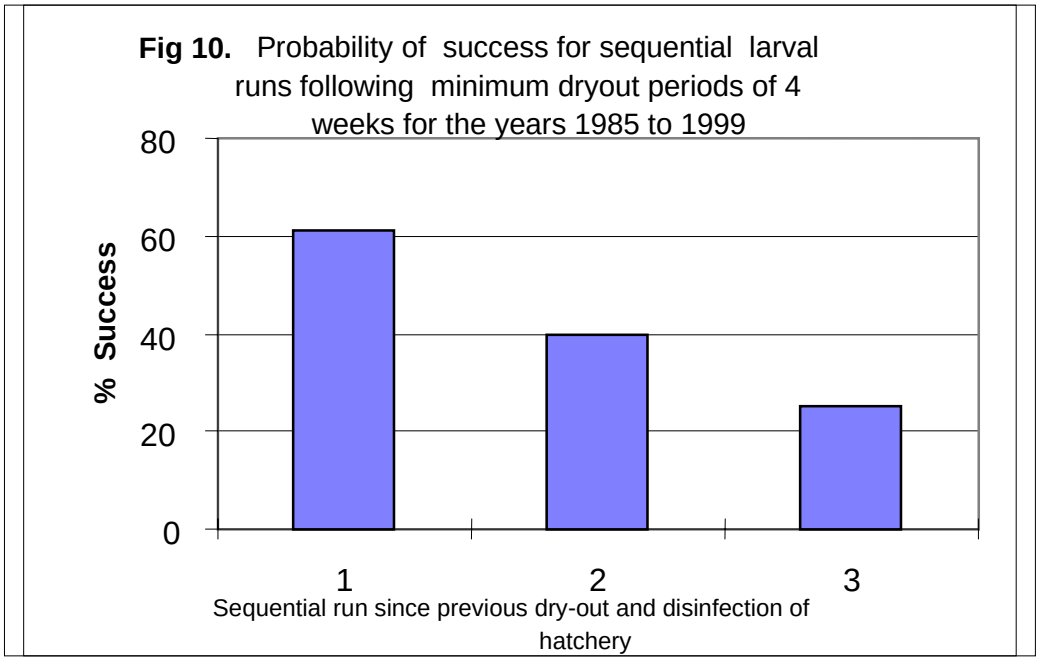


Fig 10. Probability of success for sequential larval runs following minimum dryout periods of 4 weeks for the years 1985 to 1999

Fig 11. Effect of time since previous hatchery run with *S.glomerata* (as opposed to disinfection and dryout) on occurence and severity of larval mass mortality disease (F = failed run)

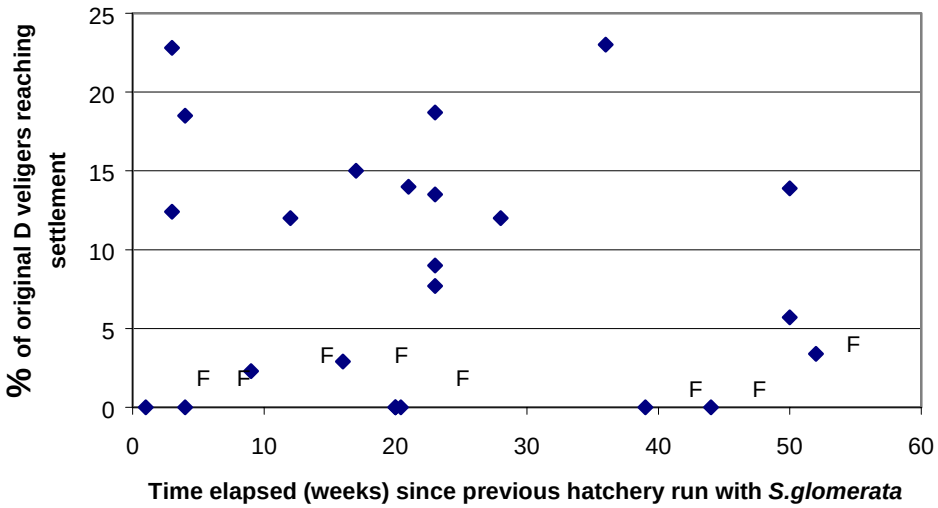
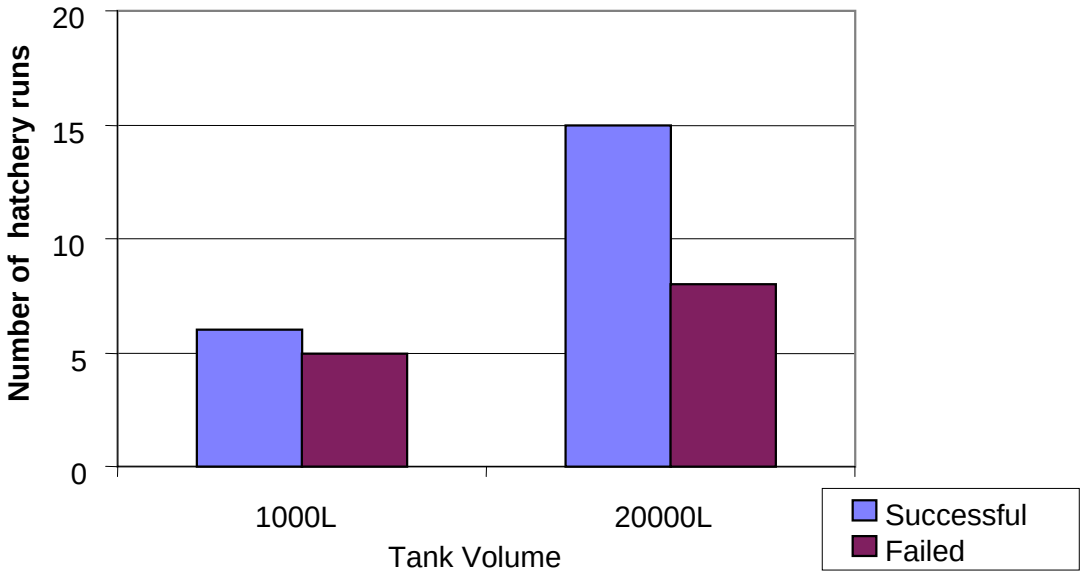


Fig 12. Effect of hatchery rearer volume on success rate of hatchery trials (new hatchery data only)



5.3 Pathology (Histopathological and micro-biological investigations)

Samples of live apparently healthy (asymptomatic) feeding larvae and moribund anorexic larvae of *S glomerata* were sent to Associate Prof. Peter Hanna (Deakin University, Victoria) in February 1994. These samples were subjected to FITC immunofluorescence tests with a panel of monoclonal antibodies that identify *Vibrio* bacteria to species level (Hanna et al., 1991). In these tests most of the seawater was removed from the larvae and then centrifuged to concentrate the bacteria for testing. Prior to testing, the larvae were sonicated for 5 seconds to open any closed shells, thereby allowing an exchange of solutions. The results of the normal *S glomerata* larvae showed that *V. alginolyticus* bacteria were present on the larval shells (see Appendix 3), but that bacteria were not detected in the water.

By contrast, the samples of diseased *S glomerata* larvae had *V. alginolyticus* present both on the shells and in the tissues and in numbers about three times greater than in asymptomatic larvae. In addition the tissues of diseased larvae often appeared to be necrosing. Test of water surrounding diseased larvae also contained considerable numbers of *V. alginolyticus*, not seen in the asymptomatic larvae. Prof. Hanna concluded that *V. alginolyticus* appears to be associated with diseased larvae. Of concern was that asymptomatic larvae also had *V. alginolyticus* present on their shells.

In May 1995, the anorexia disease occurred in *S glomerata* larvae. This outbreak was followed by succession of six more disease episodes involving a batch of clam larvae (*Tapes dorsatus*) and a batch of scallop (*Pecten fumatus*) larvae as well as further batches of *S. glomerata* larvae. Bacteriological staining (Sudan black) of live mounts and independent histopathological examination of diseased *S. glomerata* larvae by Dick Callinan (NSW Fisheries) showed patchy degeneration and necrosis of the alimentary tract epithelial cells in 4 to 5 day old larvae.

Diseased *S. glomerata* larvae from both genetically selected and control non selected breeding stock exhibiting classic symptoms of anorexia and stalled growth were sent to Prof. Hanna in May 1996. These were again subjected to FITC – immunofluorescence tests as described above and found to carry at least 3 main types of bacteria including *Vibrio alginolyticus* but low numbers precluded a definite diagnosis.

Diseased larvae were sent to Dr Judith Handlinger, and subjected to conventional histopathology and electron microscopy examination. These failed to detect the presence of a herpes virus disease of a type recently found to have caused total mortality of 5 to 7 day old clam larvae in a Tasmanian hatchery (Handlinger, pers. comm). Dr Handlinger also reported that gut epithelium cells of these larvae, were characterised by irregular long brush borders with some sloughing and apparent vacuolation. (Vacuolation of the gut epithelium, illustrated in Appendix 4, was also reported by Dick Callinan and by Dr Alex Hyatt).

Additional consignments of diseased larvae were sent to Dr Mike Hine (NIWA, NZ) and to Dr Alex Hyatt at AAHL for EM examination for possible viral infection. Again results failed to implicate viruses as a cause of the disease. Likewise Dr Tristan Renault at the IFREMER research institute La Tremblade, France and Dr Mike Hine (NIWA, New Zealand) were unable to confirm the presence of a herpes virus known to cause a similar disease symptoms in Pacific oysters.

5.4 Experimental Investigations

5.4.1 Challenge tests with Vibrio isolates

In June 1996, bacteriological plating of rearing water and of diseased larvae on marine agar and Harris media was used to isolate three *Vibrio* species. All three were propagated, purified (two passages), identified by Dr Lachlan Harris (Oonoonba Veterinary Research Station (Qld. DPI), successfully cryopreserved and used in challenge tests. The three species identified as *V. spendidus, V. mediterranei and* Vibrio sp. phenon 10/85 did not include *Vibrio alginolyticus* which had been persistently associated with previous disease outbreaks of scallop larvae and spat at the PSFC. Indeed none of the bacterial species isolated were generally recognised as causing disease symptoms of the type encountered and none were clearly capable of generating disease when used in subsequent challenge tests at 10^6/ml.

5.4.2 Experiment to further elucidate the suspected involvement of an infective agent(s)

This experiment was conducted in the PSFC hatchery in December 1997 in parallel with a commercial scale *S. glomerata* hatchery production operation. Four experimental rearing treatments were run in a total of twelve (3 replicates per treatment) 80L cylindro-conical polyethylene rearing vessels. Four of the 80L containers were accommodated in each of three, 1,000L water baths operating at 24°C with one replicate per treatment located (randomised for position) in each water bath.

To minimise the probability of inadvertent transmission of a possible disease agent(s) between the 4 treatments:

- each rearing vessel was fitted with drip, spray and aerosol proof lid.
- harvesting equipment was thoroughly cleaned, chemically disinfected with 100 ppm active chlorine solution and rinsed.
- all vessels including airlines were thoroughly cleaned between uses, chemically disinfected with chlorine solution, rinsed with bore water and air-dried between successive uses.

The four experimental treatments comprised seawater subjected to a range of preparation steps as follows:

Treatment 1 (control) EDTA (1 mg/L) treated seawater that had been trucked as usual from local beaches at either Anna Bay or Shoal Bay and stored for 7 to 14 days in outdoor 50 000 L tanks before being pumped to the hatchery via successive polyester felt filter socks with a nominal pore sizes of 5 and 1 microns respectively.

Treatment 2 As for Treatment 1 but passed through 2 x 1µm (nominal) wound cartridge depth filters mounted in series .

Treatment 3 As in Treatment 2 with additional passage through activated charcoal to remove possible bio-toxins.

Treatment 4 As for Treatment 2 except that fresh seawater was sourced from the abalone R&D facility at Tomaree Headland and conveyed in a sterilised 350L fish transporter.

Fertilised eggs from the same batch used in the above hatchery experiment were also reared in quarantine at the abalone research facility at Tomaree Head. Seawater used to rear these larvae in a 1000L vessel was treated in the same manner as in Treatment 4 of the bivalve hatchery trial.

All other hatchery rearing protocols including rearing temperature of 24°C and feeding regimes were the same as usually applied to *S. glomerata* larvae at the PSFC.

5.4.3 Results

Larvae in all four experimental treatments (in common with the commercial batch of larvae reared in a 20,000 L vessel) succumbed to the wasting diseases (ubiquitous cessation of feeding and growth) by day 5. By contrast, larvae reared in a 1,000L vessel in quarantine at the Tomaree Head facility remained asymptomatic, feeding, growing and surviving normally to and beyond metamorphosis. (Frankish et al., 1991).

Diseased larvae subjected to SEM, TEM by Dr Alex Hyatt and conventional light microscopy histopathological examination by Dr Handlinger failed to detect significant numbers of bacterial, protozoan or viral agents in or on affected larvae, nor indeed any other obvious histopathology.

5.4.4 Conclusions

These results in conjunction with other evidence already presented indicate that the anorexia disease of *S. glomerata* larvae:

- Is not vertically transmitted
- Is not caused by seawater quality, dietary or stock quality factors and cannot be ascribed to invasive disease agents.

It is therefore probable that the cause of this wasting disease is cytotoxic and appetite suppressing compounds (exotoxins), probably *in situ* bacterial in origin and probably associated with bacteria persisting on wet surfaces within the bivalve hatchery or associated seawater storage and plumbing system at the PSFC between consecutive production cycles.

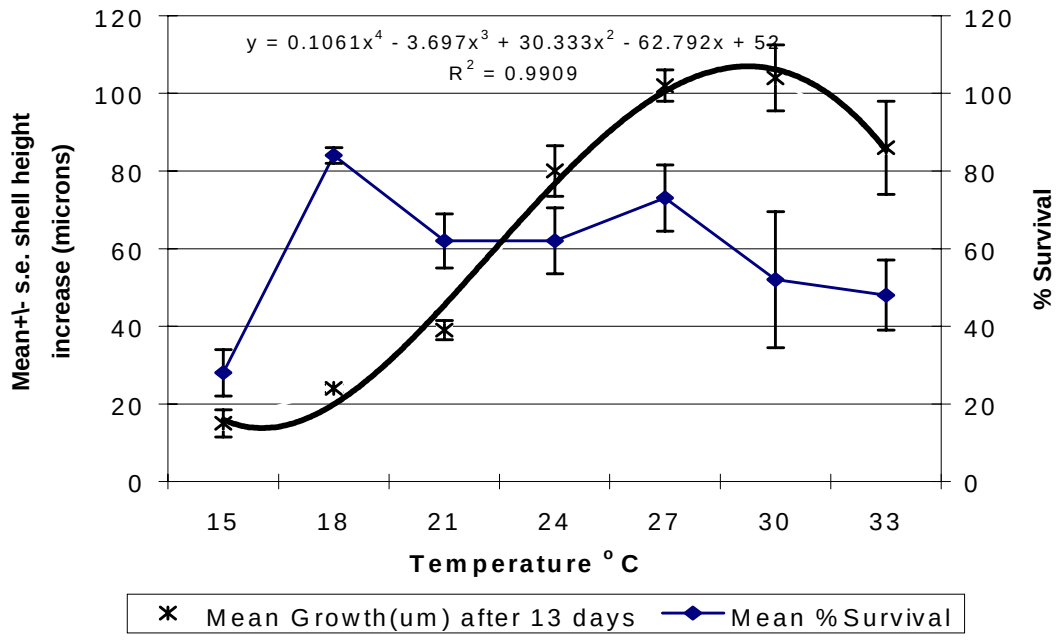
5.5 Disease prevention through optimised temperature regimes for gamete storage, incubation and larval rearing of *S. glomerata* – Summary of results

Experiment 1 – Evaluation of the effects of storage temperature on sperm motility.
To ensure retention of a high degree of motility, sperm should be stored at or below 15^0C and preferably used within an hour of collection.

Experiment 2 - Effect of temperature and storage time on fertilisation.
Storage periods for eggs over which high rates of fertilisation (>90%) are assured decrease from 2h at 15^0C to less than 30 mins at 30^0C.

Experiment 3 – Effect of temperature (Fig 13) on growth and survival of larvae.
Optimum larval rearing temperature was found to be 29^0 C which is 5^0C higher than the routinely practised rearing temperature of 24^0C

Fig 13. Effect of temperature on growth and survival of *S.glomerata* larvae



5.6 Use of Probiotics

From 1994 to 1996 intellectual property constraints prevented importation and evaluation of an array of probiotic bacteria shown by colleagues in the USA (Dr Philippe Douillet, University of Texas at Austin) and Japan (Nogami and Maeda) to be effective in combating vibriosis in marine larvae including those of another rock oyster, *Crassostera virginica* (Douillet, 1994). An opportunity to evaluate probiotic bacteria for combating the suspected cause (exotoxin forming bacteria) of mass mortality diseases of larval and juvenile *S. glomerata*, arose in 1998 from the work of Dr Lewis Gibson and post graduate students at the Dept of Cell Biology at the University of Technology (UTS), Sydney. Dr. Gibson's team had identified several strains of *Aeromonas* bacteria with demonstrable inhibitory effects on marine *Vibrio* bacteria of known pathogenicity to finfish and invertebrate larvae including those of the Pacific oyster (Gibson et al., 1998).

Preliminary research to evaluate probiotics in combating larval disease was conducted in collaboration with Dr Lewis Gibson and BSc Hons. student Mr Edward McGregor. Eighteen species/strains of bacteria isolated from seawater and from surfaces of vessels containing diseased larvae were identified as either *Vibrio* or *Aeromonas* species. All but one strain were found sensitive to tetracycline and thirteen were significantly inhibited by a probiotic strain of Aeromonas (UTS-1999) bacteria (McGregor, 1999). A 3x3 factorial design experiment involving the use of seawater from three alternative sources, namely:

1. normally sourced, stored and 1μm filtered seawater
2. contaminated seawater used to rear diseased larvae
3. stored seawater sourced from the marine fish hatchery and prefiltered to 1 μm

These seawater sources were used alone or in combination with either a probiotic bacteria (UTSA 1999) at 10^3 cells/mL, or an antibiotic (chloramphenicol at 5 mg/L) of broad spectrum efficacy in inhibiting vibriosis in bivalve larvae (Lodeiros et al., 1987; Jeanthon et al., 1988). Five replicates for each of the nine treatments comprised 250 ml conical flasks containing 150 ml of seawater stocked with 1 day old *S. glomerata* larvae at 5/ml and held in a shaker incubator at 25±1°C for 8 days.

Results presented in Figs 14, 15 and 16) showed that:

- Contaminated water reduced food ingestion and increased mortality compared with other treatments.
- The antibiotic generally improved food consumption reduced rate of mortality and marginally improved growth rate.
- Use of UTSA 1999 did not generally improve food ingestion or growth rate but may have marginally reduced mortality although not significantly.

Fig 14. Effect of seawater source,an antibiotic and a probiotic bacteria (UTSA 199) on gut fullness in 8 day old *S. glomerata* larvae

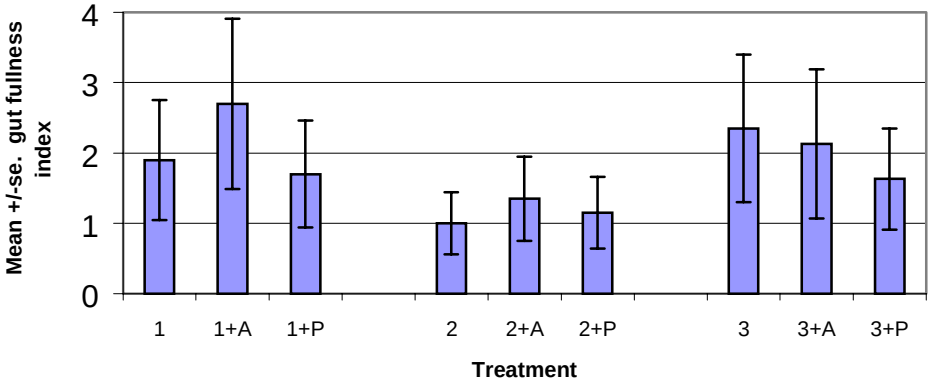


Fig 15. Effect of seawater source, an antibiotibic and a probiotic bacterium (UTSA 199) on survival to day 8 of *S. glomerata* larvae

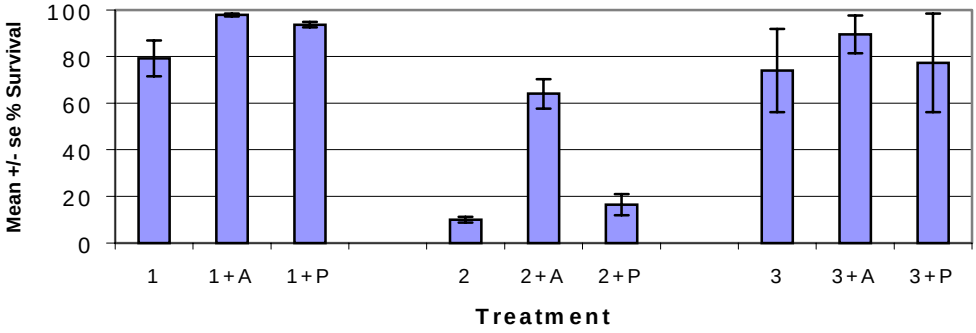
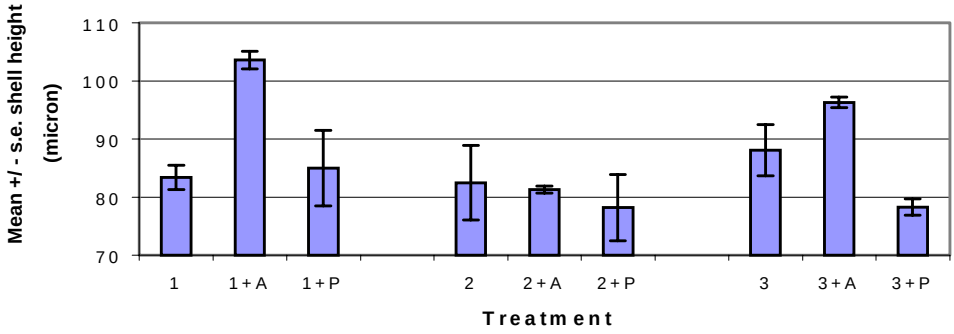


Fig 16. Effect of seawater source, an antibiotic and a probiotic bacteria (UTSA199) on growth of *S. glomerata* larvae to day 8



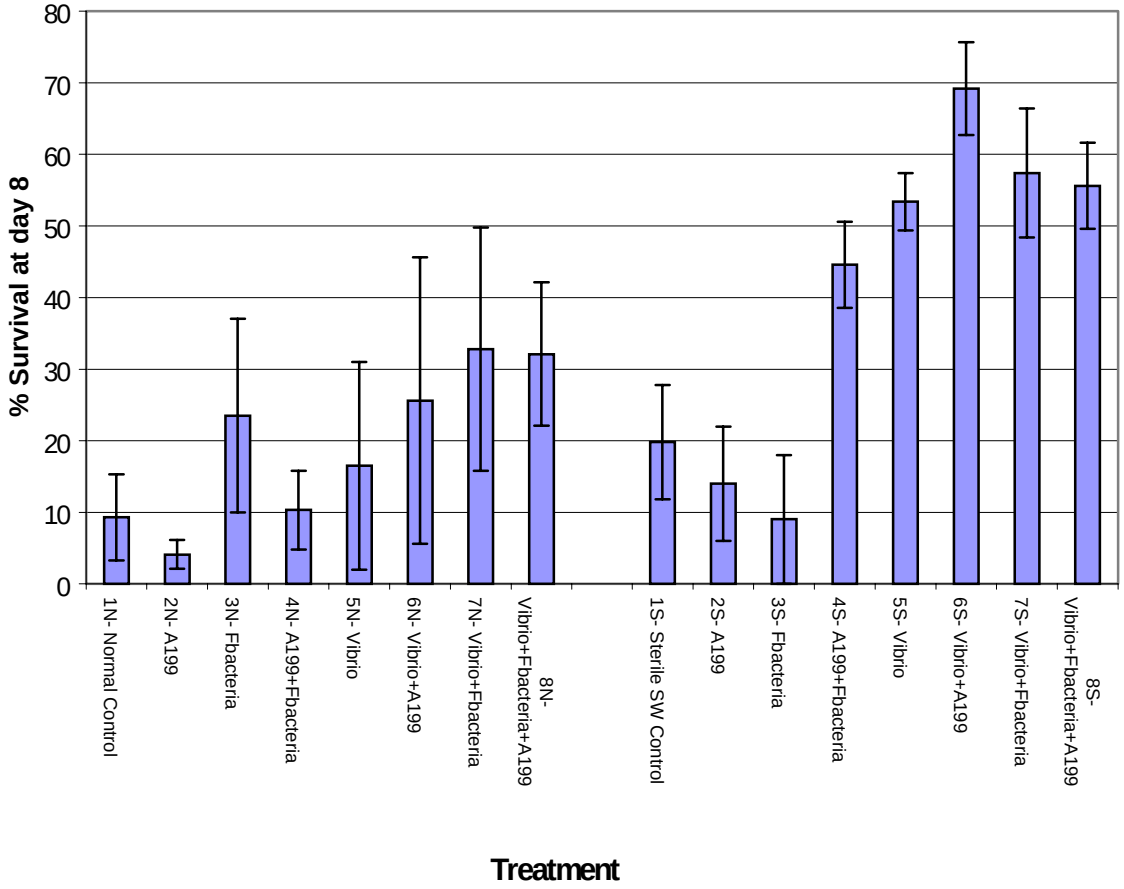
Seawater source: 1 = mollusc hatchery store; 2 = contaminated; 3 = marine fish hatchery
Additive: A = antibiotic; P = probiotic

These results together with those of the earlier research of Gibson et al., (1998) with *C. gigas* were sufficiently encouraging to prompt a successful application for an ARC/SPIRT grant to develop probiotic techniques to combat mass mortality diseases of larval and juvenile *S. glomerata.* Dr Gibson serves as chief investigator; with Dr Mike Heasman and Dr Tony Maurice as co-investigators and NSW Fisheries as a commercial partner. BSc Hons. graduate Mr Cheok Tan was selected as the successful PhD candidate for the project in December 1999. The first experiment, to evaluate the use of several bacteria as probiotics to prevent and combat the larval disease was conducted in March 2000.

In the inaugural 2x8 design experiment of the project, seven bacterial flora manipulation treatments plus a control were applied in combination with two methods of pre-treating seawater used to rear *S glomerata* larvae for 8 days post hatch. The bacterial flora treatments comprised the pre-inoculation of rearing water with single, paired or all combinations of three different bacteria. The first of these was a UTSA 1999, previously shown by Gibson et al. (1998) to counteract pathogenic strains of *Vibrio tubiashi* when applied as a challenge to Pacific oyster (*Crassostera gigas)* larvae. The second was, a bacterium previously shown to be non pathogenic and of substantial nutritional value (a food bacteria) to *S glomerata* larvae by Nell et al. (1993). The third bacteria was an archived strain of *Vibrio alginolyticus* held by UTS that was added as a challenge rather than as an alternative probiotic.

Results (Fig 17) showed that sterilization (autoclaving) of sea water followed by re-inoculation with one or a combination of the 3 different selected species of bacteria raised survival of 8 day old *S, glomerata* larvae from a base level of only 9% (typical of disease episodes) in control larvae reared in conventionally treated sea water (stored for ≥7 days and 1μm filtered), to a range of 45 to 68%. The latter survival rates are among the highest achieved with *S. glomerata* larvae. Benefits conferred by sterilization and re-inoculation of seawater with non pathogenic bacteria were equally dramatic in relation to enhanced larval growth (Fig 18).

Fig 17. Effect of seawater sterilization and added bacteria on mean+\- s.e. % survival of day 8 *S. glomerata* larvae [note: prefix N=stored, 1um filtered seawater; prefix S=sterilized (autoclaved) seawater]



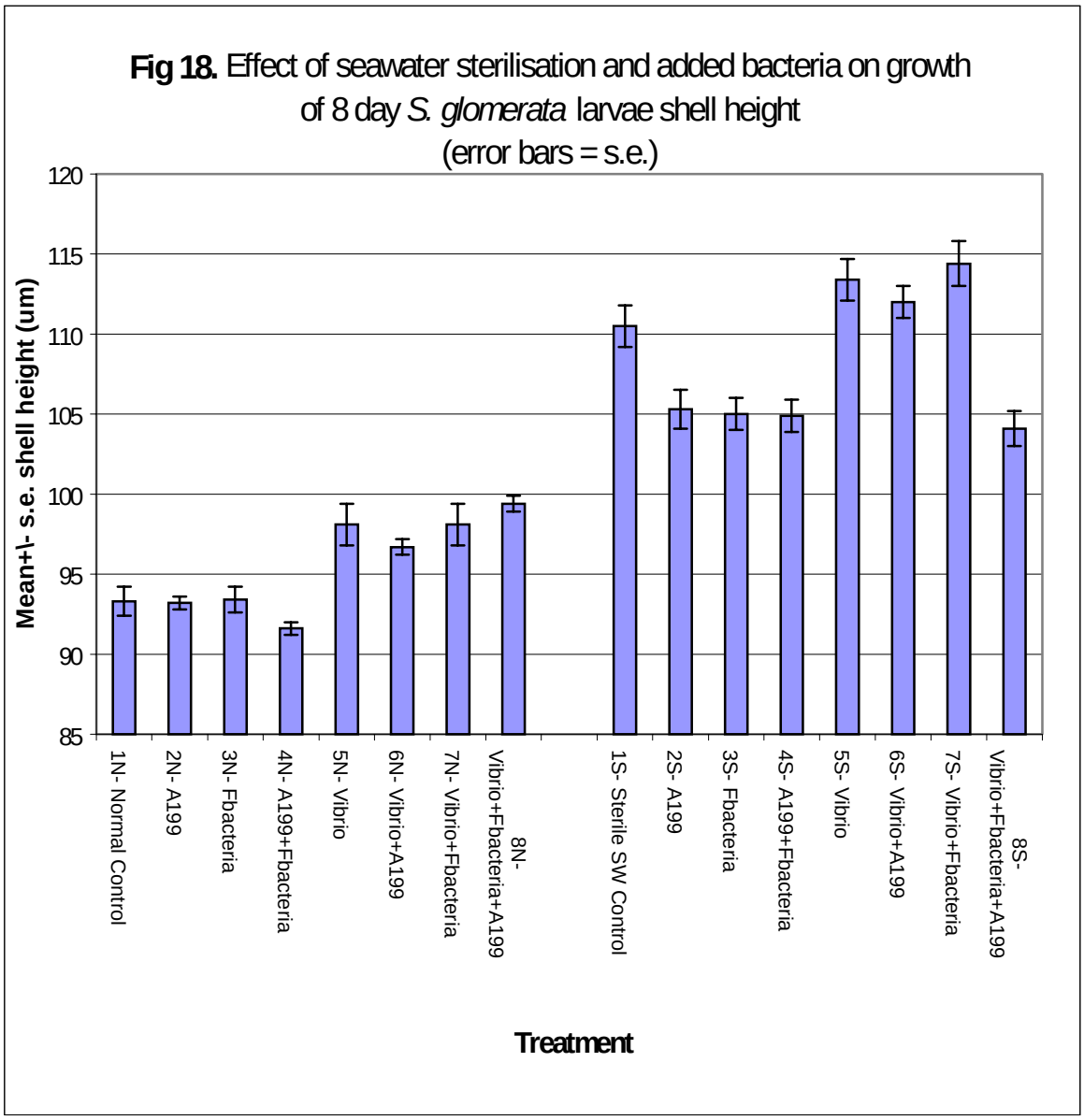


Fig 18. Effect of seawater sterilisation and added bacteria on growth of 8 day S. glomerata larvae shell height (error bars = s.e.)
Mean+\- s.e. shell height (um)
120
115
110
105
100
95
90
85
1N- Normal Control
2N- A199
3N- Fbacteria
4N- A199+Fbacteria
5N- Vibrio
6N- Vibrio+A199
7N- Vibrio+Fbacteria
8N- Vibrio+Fbacteria+A199
1S- Sterile SW Control
2S- A199
3S- Fbacteria
4S- A199+Fbacteria
5S- Vibrio
6S- Vibrio+A199
7S- Vibrio+Fbacteria
8S- Vibrio+Fbacteria+A199
Treatment

6.0 INVESTIGATION OF MASS MORTALITY SYNDROME OF JUVENILE *S. GLOMERATA*

6.1 Epidemiological Investigations

6.1.1 Background

As with the larval mortality disease, all available hatchery data (Appendix 2) were extracted from diary records dating from September 1985. Reports compiled by and by Goard (1990), Nell et al. (1991) and by Frankish et al. (1991) were also reviewed in the light of more recent experience and information gathered. A search of published literature was also conducted in an attempt to uncover disease case- histories and research findings bearing close apparent resemblance to those afflicting spat of *S. glomerata*.

In 1987, the still current down-weller set system was installed at the PSFC. This incorporated the use of ground and graded (250 – 500 μm) scallop shell culch within 450 mm diameter 210 μm mesh screens each holding 100 000 to 200 000 single seed spat. Using this system to maintain single seed and to prevent doubles or triples etc., spat are separated from larvae using a 350 μm mesh screen. After approximately two weeks, spat are large enough to be retained on a 350 μm mesh and are removed from this hatchery system and placed in outdoor upwellers. From 1987, passive upweller systems located either in the field and fed raw estuarine water or on site at the PSFC and fed re-circulated brackish pond water, have been used.

While the introduction of ground scallop shell single seed settlement culch in conjunction with a Bayes (1981) downweller nursery system enabled consistent large scale (>1 million) production of single seed *S. glomerata* , these large batches often suffered intermittent mass mortalities. Over the period 1987 to 1990, these mass mortalities first manifest 8 to 30 days after the larvae were introduced to the settlement system. Apparently healthy spat moved from the hatchery at the PSFC to upwellers in different estuaries also experienced sudden and severe mortalities within 5 to 7 days. These outbreaks always coincided with mortality of spat remaining in the hatchery. On one occasion however spat, some of which were moved out of the hatchery 3-4 days after settlement (size >350 μm), subsequently survived in the upwellers while those retained in the hatchery died.

The investigation of Nell et al. (1991) evaluated intensified hatchery hygiene protocols, especially increased rinsing of spat and disinfection of settling tanks and equipment. The latter did not significantly reduce total bacteria, presumptive *Vibrio* bacteria, or the subsequent survival (14%) of spat relative to controls. It did however reveal very large concentrations (14 to 18 $x10^4$ g^{-1}) of presumptive *Vibrio* bacteria on conventional ground scallop shell culch used to induce settlement of *S. glomerata* pediveligers as single spat.

In an associated report to that of Nell et al. (1991), Goard (1990) made the following key observations:

- mortalities occur suddenly and severely, (up to 95% in 36 hours) in spat that show no obvious signs of stress, are actively feeding and producing good quantities of faeces, have dark coloured guts and are exhibiting good shell growth"
- survivors on-grow normally during and after rapid pulses of mortality
- Spat set on the sides of downweller screens and not removed appear to be least effected.

In reference to an experimental investigation of the disease, Goard (1990) also stated that all spat were on 670 µm and 500 µm screens exhibited mortality one day after their first multiple grading and counting with cumulative mortality increasing to approximately 90% over the next 6 days. By contrast spat that had set on flat fibrous cement slats that had been laid flat on the screens on top of beds of scallop shell, or on the smooth plastic walls of the nursery screens, suffered negligible mortality. While there was no absolute proof that bacteria cause or contribute to spat mortalities, a bacterial linkage to the disease was supported by the far superior survival of spat that had set on the slats and walls.

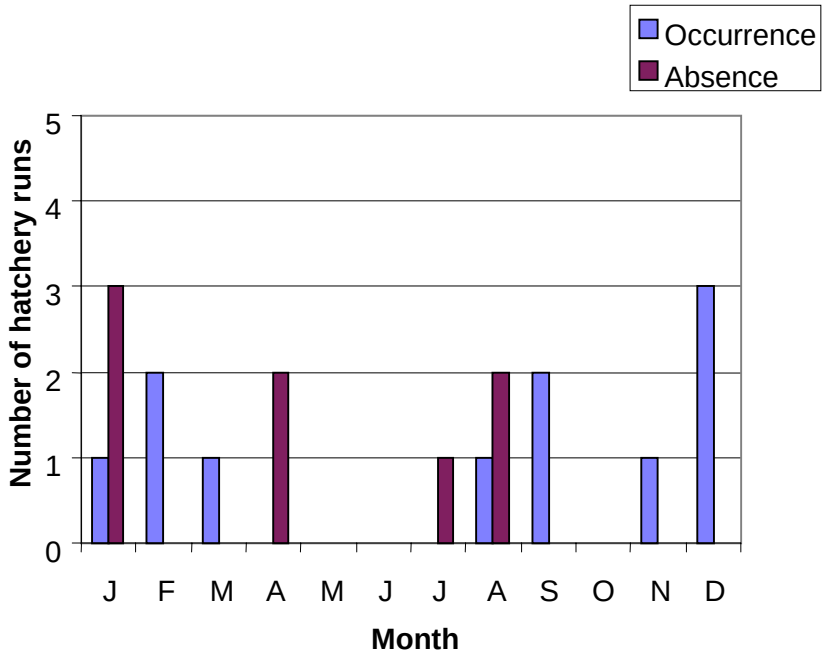
6.1.2 Results

Evaluation and re-evaluation of hatchery records 1988-2000

Examination of general hatchery records useful epidemiological information extracted from records for operations conducted in the new hatchery 1988 to 1999 were as follows:

1. *Seasonal Factors.* In contrast to the larval disease, no seasonal trends in the occurrence or absence of mass mortality of juvenile *S. glomerata* were apparent from these data (Fig 19).

Fig 19. Seasonal occurrence or absence of mass spat mortality episodes 1988 to 1999 (n=19)



2. *Effect of larval rearing tank size*

Also in contrast with the larval disease, juvenile *S. glomerata* reared as larvae in commercial scale 20 000 litre tanks experienced much higher relative incidence of mass mortality (9 out of 11 documented cases) than their counter parts reared in much smaller 1000 litre tanks (only 1 out of 6 cases).

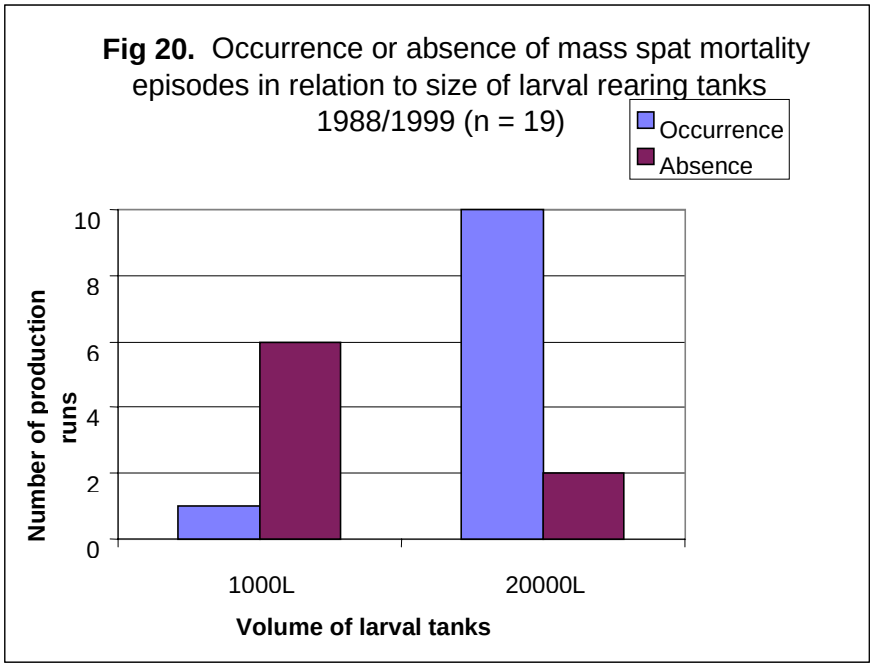


Fig 20. Occurrence or absence of mass spat mortality episodes in relation to size of larval rearing tanks 1988/1999 (n = 19)

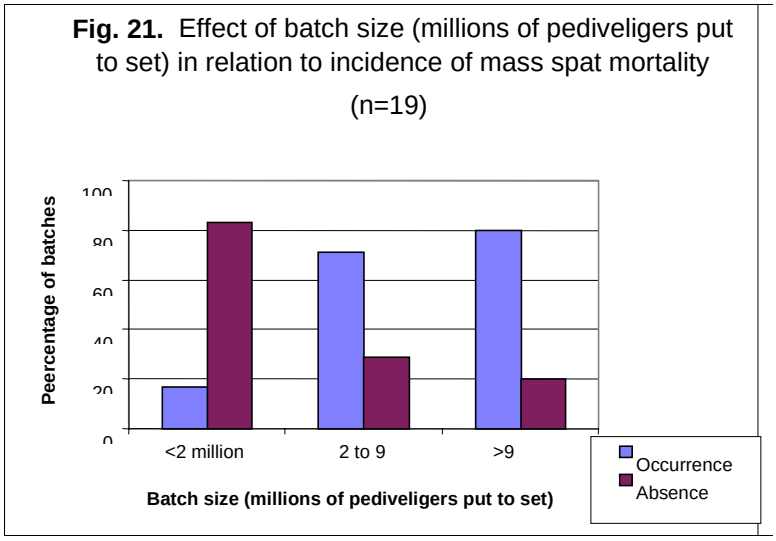


Fig. 21. Effect of batch size (millions of pediveligers put to set) in relation to incidence of mass spat mortality (n=19)

A possible explanation for this unexpected observation was density factors associated with larger batch sizes of juveniles reared in 20 000 litre tanks. This explanation was supported by data collected from the same 17 batches of spat referred to in Fig 20, that were pooled according to batch size and regardless of the volume of the larval tank used. These data (Fig 21) strongly suggest that the probability of mass spat mortality episodes did indeed increase with the absolute size of batches and probably was not directly influenced by larval tank volume.

Intensive monitoring of spat during epiozooitics

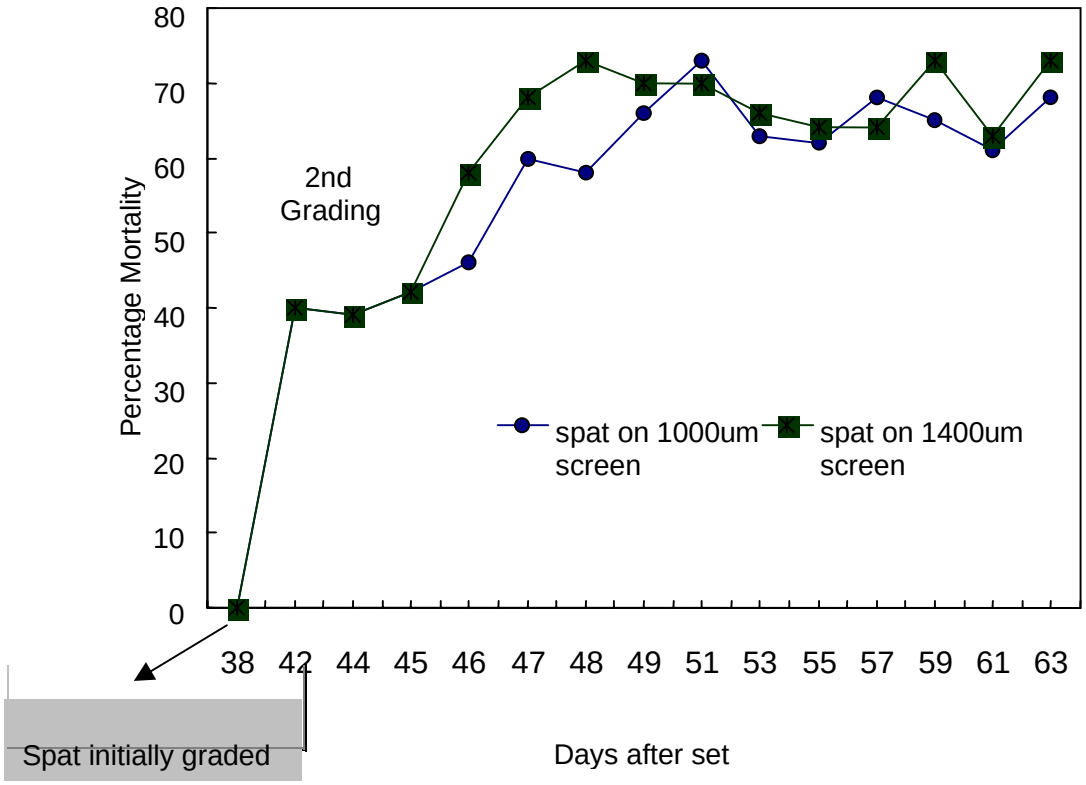
Case History 1- Production of triploid of *S. glomerata*- February 1994

The first recurrence of the Mass Mortality Syndrome in more than three years was experienced during the February 1994 hatchery production run. No deaths or any other signs of disease were observed prior to the detection of mass mortality rates of 40% and 53% respectively in preserved diploid and triploid of *S. glomerata* spat sampled on April 11(day 42 in Fig 22). By this time all

dead spat were free of tissue remnants suggesting that all deaths had occurred as a short sharp pulse within four days of initial multiple grading and counting (day 38 in Fig 22).

The mortality data presented in Fig 22 are suggestive of a possible link between the mass mortality syndrome and stress and physical damage sustained by small spat during protracted sieve grading and counting using volumetric methods. Growth rates of surviving spat, as in previous epizootics discussed above (Goard, 1990 and Nell et al., 1991), appeared to continue normally both during and beyond periods of sudden and severe mortality.

Fig 22. Mortality data *S.glomerata* mass mortality episode Feb.94



Case History 2- Monitoring of early juvenile stock produced during the course of production of 3rd generation mass selection lines of *S. glomerata*-in January and February 1995

In response to the recurrence of the mass mortality episode in February 1994 and possible implication of disturbance and trauma sustained in grading and counting, the progressive growth and mortality of broods of spat from 6 separate breeding lines of *S. glomerata* were closely and frequently monitored prior and subsequent to successive grading and volumetric counting operations in January and February 1995. These grading and counting operations were conducted 12 days apart and were applied as juvenile stock attained mean shell heights in the order of 600 to 800 microns and again at 900 to 1100 microns in accordance with what was then standard practice.

As illustrated in Figs 23 to 24, up until days 9 to 11 post-set (18th and 19th January) when all 6 genetic lines and grades thereof in the size range 500 to 800 micron were re-graded, cumulative mortality remained below 8%. However over the 1st to 3rd days after grading it rose markedly (15 to 38%) across all breeding lines in the smaller grade spat and across three of the six breeding lines

in the case of larger grade spat that had already been transferred from the hatchery to the field upweller nursery at Wanda Head, Port Stephens.

During the 9 days between successive gradings on the 18th and 19th and on the 29th and 30th January, a major disparity developed between cumulative mortality in the four genetically selected lines of spat and the two control lines of spat those produced from non genetically selected parent stock. This disparity was consistent across the larger and smaller size grades but was most pronounced in relation to the larger grade. By the 11th February, cumulative mortality for larger spat across all four genetically selected lines remained at or below 10% while the two control non selected lines of spat exhibited mortality rates of 61 and 87%.

These results in conjunction with histopathological evidence presented below, supported the initial hypothesis, that mass mortality episodes occur as pulse events that are caused by bacterial infections triggered by such things as trauma and physiological stress sustained by small spat during mechanical sieving. As observed in previous episodes, growth rate of surviving spat did not appear to be checked prior to, during or following pulse mortality events i.e. the disease appears to manifest as an all (rapid death of afflicted spat) or nothing (no sub-lethal effects on surviving spat) phenomenon.

An additional pertinent observation of growth rate data presented in Figs 25 and 26 is that good growth rates of 45 to 50 micron/day were exhibited by all breeding lines of both size grades from the time of settlement to the time of transfer to the field nursery 9 days later. Once in the field nursery growth rate slowed to a universally low rate of 20 to 25 microns/day and may have reflected dietary or density related suppression of growth that otherwise would be expected to be in the order of 50 to 100 microns /day under the high (23 to 25°C) ambient summer temperatures prevailing at the time.

A possible explanation for the markedly lower rates of mortality sustained by genetically selected lines of spat was that four generations of selection for fast growth and survival has inadvertently conferred some genetic resistance to the mass mortality disease. On the other hand, slight inadvertent differences in handling or density factors may have been responsible. The only way to confirm genetic resistance was to gather further evidence during future selective breeding operations.

Fig 23. % Mortality for the six breeding lines in the mass selection experiment (smaller grade) Note: arrows indicate dates of grading and counting

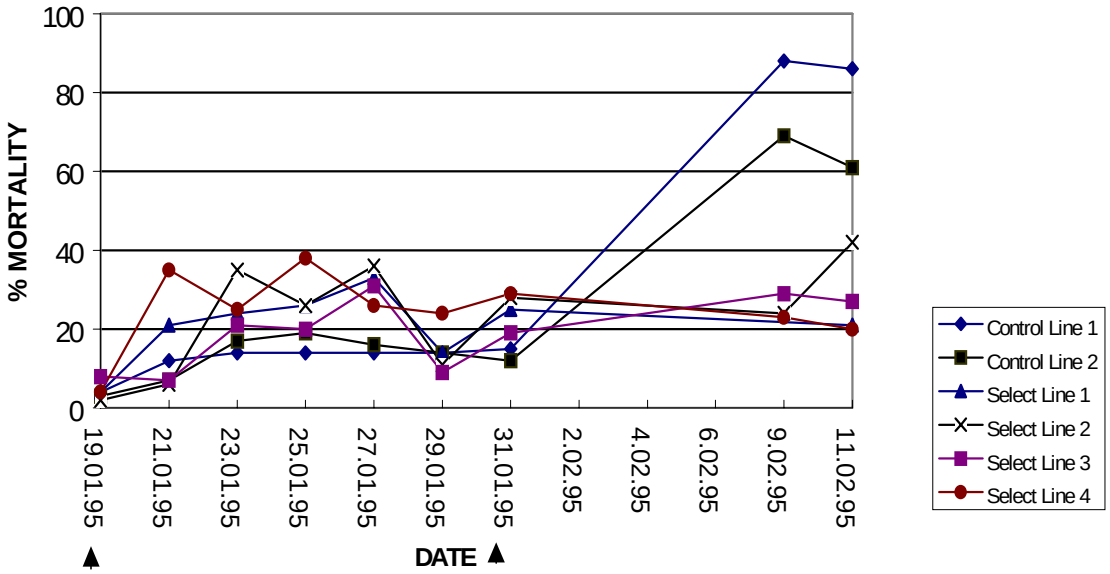
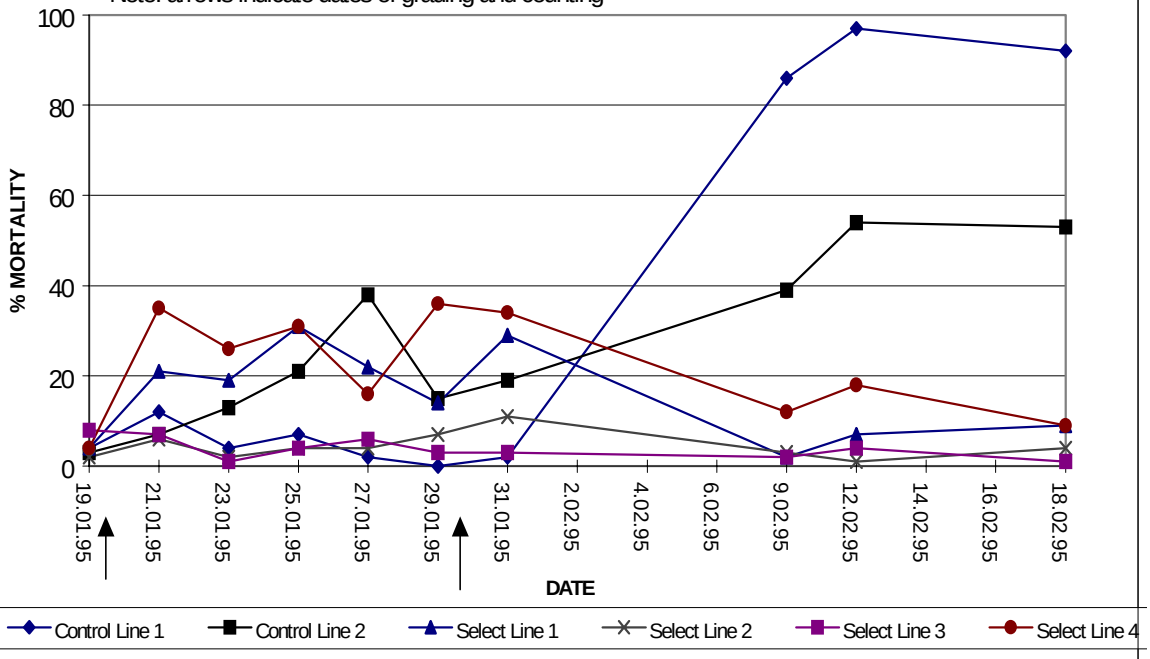


Fig. 24. % Mortality for the six breeding lines in the mass selection experiment (larger grade) Note: arrows indicate dates of grading and counting



Case History 3- Monitoring of juvenile stock produced during the course of a hatchery operation to produce a batch of triploid spat for sale to farmers in late 1998 /early 1999.

A batch of triploid *S glomerata* spat were put to set on the 10 to 14th October 1998. These were retained in the hatchery then transferred to the Wanda Head field upweller nursery. The health of these stock was monitored every few days. Significant numbers of dead or dying (gaping) spat were first detected following a very protracted period of low temperature suppressed growth. Confirmation that the batch had suffered a mass mortality episode was confirmed over the following week as daily estimates of cumulative mortality rapidly rose to about 70%

To check whether or not mass mortality had occurred as short duration pulse and that those spat that did not die continued to grow normally, the status (live or dead) and size frequency distribution of 300 spat randomly sampled from each of three separate upweller screens, was determined on the 18th of December and again 17 days later on the 4th January 1999.

The initial mean± s.e. percent of dead spat on 18th of December (Fig 27) was 79.8±4.3%. This remained unchanged when re-assessed as 73.8± 0.8% on the 4th January (see Fig 28), indicating that no additional mortality had occurred over the interim. Likewise, mean± se. shell height of dead spat on the 4th January (869± 8 μm) was almost identical as on 18th December (849±7 μm). By contrast, mean ± se. shell height of live spat had increased from 1390± 29 μm to 2490± 46 μm over the 17 days. This constituted a very respectable mean growth rate of about 80 μm/day and probably reflected reduced densities of spat on the upweller screens following grading and the massive reduction of densities created by the mass mortality.

Fig 25. Effect of breeding lines and handling on growth of *S. glomerata* (smaller grade)

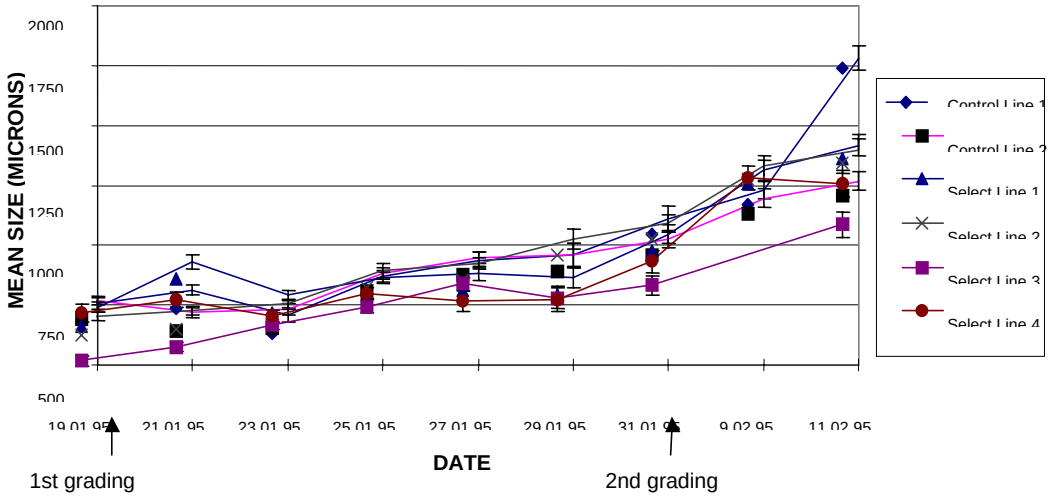


Fig 26. Effect of breeding lines and of grading and counting operations on juvenile *S glomerata* (larger grade stock)

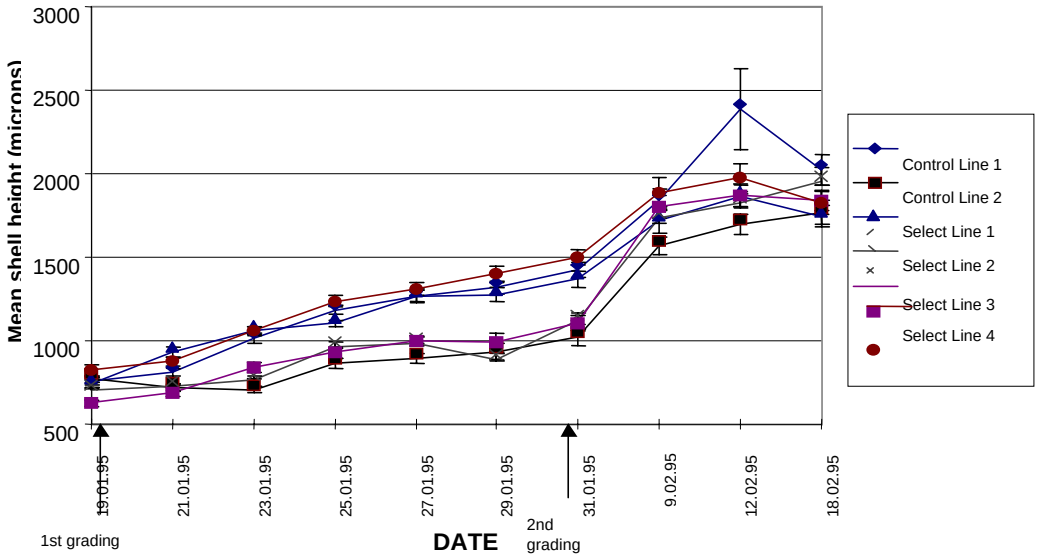


Fig 27. Initial size frequency distribution of dead and live *S.glomerata* spat

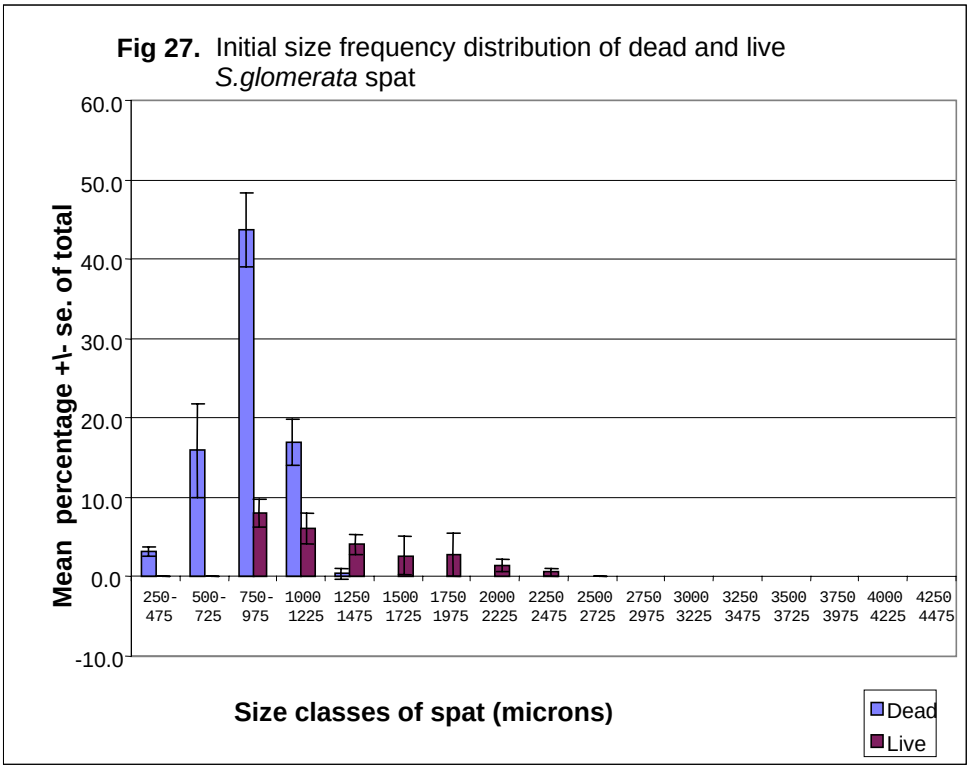
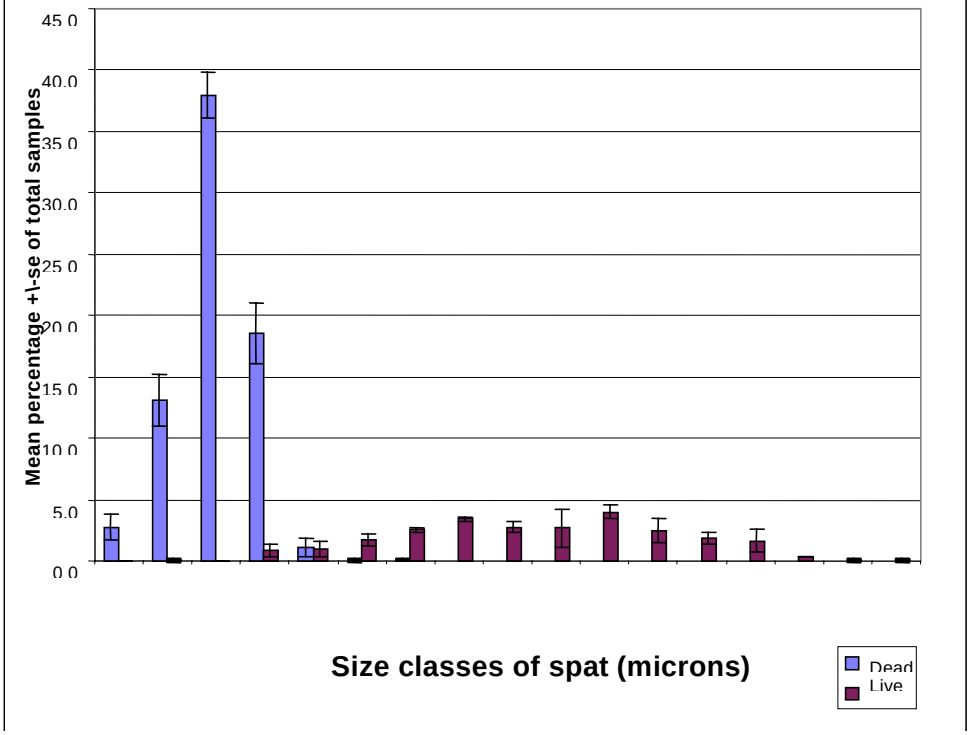


Fig 28. Size Frequency distrbution of dead and live spat 17 days later



6.2 Pathology

6.2.1 Background

In February 1994 (triploid S *glomerata* project) and again in December 1994 (mass genetic selection project) serial sampling was made of all size grades of spat that sustained mass mortality during routine hatchery production cycles. In both cases standard larval rearing, settlement on scallop shell and downweller/upweller nursery techniques described by Frankish et al., (1991) were employed. Sampled spat were fixed and stored in either 5% formal seawater or 3% each of glutaldehyde and paraformaldehyde in seawater for Scanning Electron Microscopy (SEM). Progressive growth and mortality data were derived from these samples and sub-samples submitted for specialist histopathological examination to Dick Callinan (NSW Fisheries Fish Pathology Unit, Wollongbar), for SEM to Dr Alex Hyatt (AAHL/CSIRO) and for specific bacteriological analysis using monoclonal antibodies that identify *Vibrio* species commonly isolated from shellfish (Chen et al., 1992) to Assoc. Prof. Peter Hanna (Deakin University, Geelong Victoria).

6.2.2 Histopathology reported by Dick Callinan

Histopathological examination of haematoxylin and eosin stained thin sections by Dick Callinan in 1994 revealed a range of conditions from asymptomatic to severely diseased individuals among mass mortality spat. These observations were consistent with pathology described by Dick Callinan for earlier epizootics and reported by Nell et al., 1991. Lesions seen in all obviously diseased individuals comprised mild to severe focal to diffuse inflammation in connective tissue (see Appendix 4). The parietal mantle surface was often severely effected with exudation of proteinaceous fluid and cell debris. Many spat showed partial dissolution of the hinge ligament associated with moderate to many bacteria of various morphological forms including filamentous. Many spat from the mass mortality batch also showed focal to locally extensive necrosis in connective tissue associated with rod shaped bacteria. No other potential causative agents were seen associated with the above lesions. Similar but less common and severe lesions to the above were seen among the batch of apparently healthy spat. Dick Callinan concluded, “It is possible that the non inflammatory changes seen in all spat mortality episodes so far examined and in apparently healthy groups, were due to bacterial toxins originating from culch and/or spat shell surfaces”.

6.2.3 Histopathology reported by Dr. Alex Hyatt (AAHL/CSIRO)

Spat from a mass mortality episode in April 1994s were examined by negative contrast electron microscopy (NCEM), by scanning electron microscopy (SEM) and by examination of ultra thin sections. All three techniques failed to reveal any viruses. Examination of the surface of spat (see Appendix 5) revealed significant erosion and the presence of large numbers of rod shaped bacteria plus prokaryotic fungal filaments and fruiting bodies.

6.2.4 Histopathology reported by Assoc. Prof. Peter Hanna (Deakin University)

S. glomerata spat sampled from an earlier apparently healthy batch reared at the PSFC in January 1993 and diseased spat sampled during a period of rapidly increasing cumulative mortality (15/4/94) was sent to Prof. Hanna with a view to identify the surface bacteria (originally identified by Dr Hyatt as cited above) using *Vibrio* species specific FITC immunofluorescence tests. Apparently healthy larvae samples showed *Vibrio alginolyticus* on their external surfaces, but not in supernatants (seawater). The diseased samples by contrast contained at least three times the

level of immunofluorescence associated with *V. alginolyticus* on the external surfaces (see Appendix 3). High numbers of *Vibrio* bacteria were found in association with extensive soft tissue lesions in diseased spat.

6.2.5 Conclusions

These findings thus collectively supported the initial favoured hypotheses of Dick Callinan reported in Nell et al. (1991) and Goard (1990) that mass mortalities of *S. glomerata* spat are associated with heavy bacterial biofouling of spat and residual scallop shell cultch in early stage spat. Toxins produced by fouling bacteria may have caused the apparently non specific connective tissue inflammation and mantle necrosis in both apparently healthy and diseased groups and subsequent systemic invasion by bacterial opportunists resulting in tissue necrosis of the diseased group.

On the basis of epidemiological evidence reported above it is also postulated that the recurrence of the mass mortality syndrome after a three year absence in 1994 was partly a consequence of at least two predisposing factors, namely:

- A failure to subject the hatchery to an annual dry-out cleaning and disinfection on the scheduled date of October 1993 and the use of stocking rates about three times recognised upper biomass limits (Goard - pers. comm.,) or downweller systems of this type. The lapse in implementing annual hatchery dry-out, cleaning and disinfection protocols was brought about by intense operational demands imposed by concurrent scallop, clam and oyster breeding research at the PSFC bivalve hatchery.
- Physiological stress and physical abrasion sustained by small (<2 mm) *S. glomerata* spat during multiple grading and volumetric counting operations in the hatchery, was also viewed as the likely trigger to such outbreaks. As already discussed above, a similar coincidence of sudden catastrophic mass mortality within one to several days of first grading and counting of spat was revealed in a re-examination of the Goard (1990) report.

6.3 Experimental Investigations

6.3.1 Spat mortality experiment 1

Investigation of effects of settlement technique mechanical grading and prophylactic use of an antibiotic on the incidence and severity of the mass mortality disease of early *S. glomerata* spat

Background and aims

This experiment was prompted by the above described results of epidemiological and histopathological evidence that:

- manifestation of the mass mortality disease of juvenile *S glomerata* is caused by exotoxins of *Vibrio* bacteria followed by fatal secondary invasive bacterial infections
- disease manifestation triggered by additional stress factors especially high accumulation of organic matter and bacteria associated with the use of ground scallop shell in settlement screens and the practice of mechanical sieve grading and volumetric counting of small delicate spat.

Experimental Design

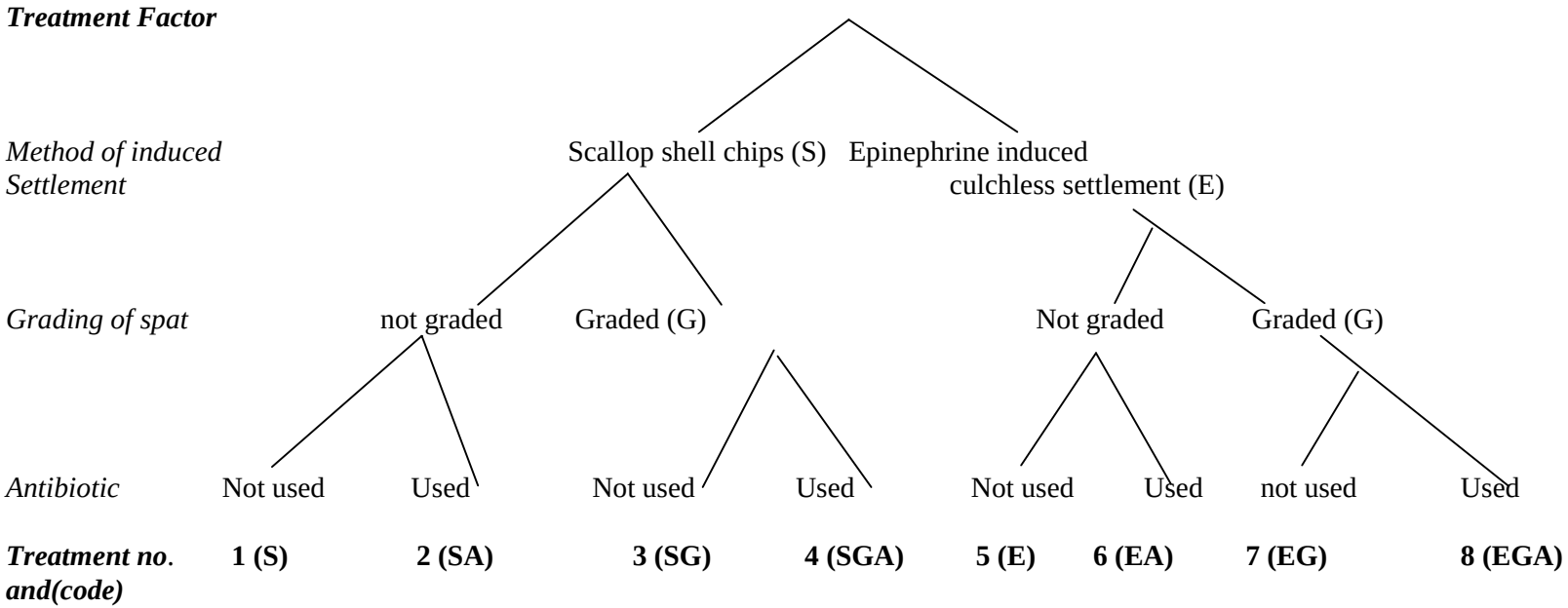
The nested factorial experimental design comprising 8 treatments represented schematically in Fig. 29 involved three factors:

1. Mode of settlement used i.e. culchless epinephrine induced settlement and metamorphosis (treatment code E) or scallop shell induced settlement and metamorphosis (treatment code S)
2. grading (treatment code G =graded)
3. use of antibiotic(treatment code A = antibiotic (chloramphenicol at 5 mg/l))

This experimental design was devised to test the hypotheses that:

- The primary cause of mass mortality of small (0.3 to 2 mm) *S. glomerata* spat is the development of large concentrations of virulent strain(s) of *Vibrio* bacteria associated with the use of ground scallop shell to produce single spat in conventional downweller and upweller nursery systems.
- Manifestation of mass mortalities (as opposed to sub-clinical disease in apparently healthy stock) depends on a triggering factor especially trauma or other stress factors such as those associated with multiple grading and volumetric counting of small *S. glomerata* spat.
- Mass mortality if caused by *Vibrio* bacteria should be prevented or ameliorated with the prophylactic use of an appropriate antibiotic namely chloramphenicol at 5 mg/l.

Fig 29. Design of experiment to test effect of settlement method, grading and prophylactic use of an antibiotic on the occurrence and severity of spat mortality



General Husbandry

Ripe broodstock oysters were supplied by a commercial *S. glomerata* farmer from Pambula on the mid south coast of NSW and strip spawned on 23rd of June 1995. Competent pediveligers were put to set on 13th and 14th of July in 6 downweller units (4 screens per unit and 50 000 larvae /screen).

In treatments 5 to 8 involving culchless (epinephrine induced) metamorphosis of spat, competent larvae were exposed to epinephrine (bitartrate salt) at 10^{-4}M in seawater for 45 minutes, on the 13th and 14th of July and again one week later on the 19th July.

For treatments 1 to 4 in which competent larvae were set onto scallop shell culch, spat were first separated from residual shell over a 350 µm mesh size screen on the 17th and 18th July and a for a second time on the 20th and 21st July. On the latter occasion, treatments 1, 2, 5 and 6 subjected to grading were additionally screened over 500 µm mesh.

Graded treatments 1, 2, 5 & 6 were graded over 350, 500 and 670 µm screens and separated size classes counted volumetrically and redeployed onto individual screens on the 24th and 25th of July. Grading for these treatments was repeated the 3rd of August using 500, 670, 1000, 1250, and 1400 µm screens.

At the conclusion of the experiment on 17th August all screens of spat from all eight experimental treatments were graded over 500, 670, 1000, 1250, 1400, 1800, 2240 and 3000 µm screens into separate size classes then counted.

Treatments 1, 3, 5 & 7 involving prophylactic use of antibiotics were continuously exposed to 5mg /L of chloramphenicol. This protocol has been found extremely reliable and is a commonly used method of combating larval Vibriosis in several bacteria prone species especially the European scallop *Pecten maximus* (Corre et al., 1993; Jeanthon et al., 1988).and the flat oyster *Ostrea edulis* (Tubiash et al., 1965; Lodeiros et al., 1987).

Results

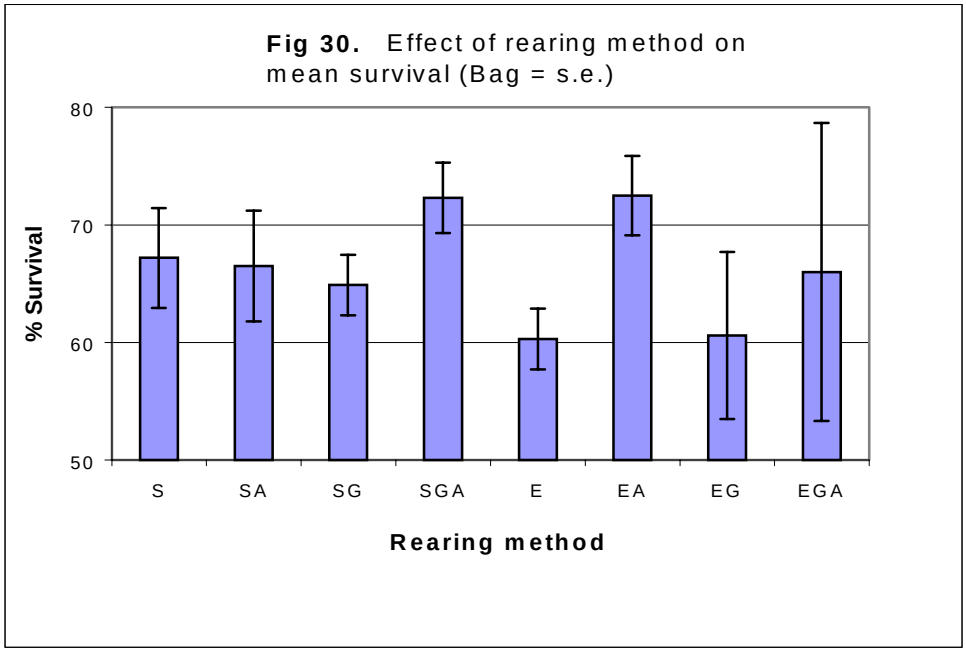
The absence of mass mortality from all of the eight experimental treatments, which may in part have been a consequence of the reintroduction of an annual disinfection and dry out of the bivalve hatchery in November 1995, precluded any additional definitive findings on the cause(s), control and prevention of the disease. Nevertheless, the following useful information was gathered on the following issues:

- the relative benefits and costs of epinephrine (culchless) induced settlement as opposed to scallop shell culch production of single spat.
- the need or otherwise to grade and count spat before reaching a size(>2 mm) at which they are apparently no longer susceptible to the mass mortality syndrome.
- general effects of prophylactic use of an antibiotic on growth and survival of spat.

At the time of the first multiple grading and counting of spat (treatments 3, 4, 7 & 8) on 24th and 25th of July, mean ± s.d. survival (Fig 30) ranged from 60.3 ±2.6 to 72.3 ± 3.0%. Survival was not influenced by method of induced settlement but was apparently enhanced by prophylactic use of chloramphenicol although differences were not statistically significant at the P 0.05 level of confidence. Mean sizes of single spat settled on scallop shell (Fig 31) were similar to those of epinephrine induced culchless settlement spat.

Comparing size frequency data for shell and epinephrine settled spat (Fig 32), it is apparent that grading of spat does not enhance average growth rate nor reduce growth variability of spat. The antibiotic did appear to marginally improve growth rate of spat settled on scallop shell but the opposite appeared true in relation to epinephrine settled spat.

Another important inadvertent finding of this experiment was that adoption of culchless settlement induced with epinephrine and omission of periodic grading of small *S. glomerata* spat drastically reduces hatchery labour requirements. Although hard evidence was not forthcoming, it is nevertheless likely that culchless settlement will reduce risks of mass mortalities by avoiding traumatic handling and elevated levels of pathogenic *Vibrio* bacteria associated with large accumulations of amounts of organic matter created by use of scallop shell culch.



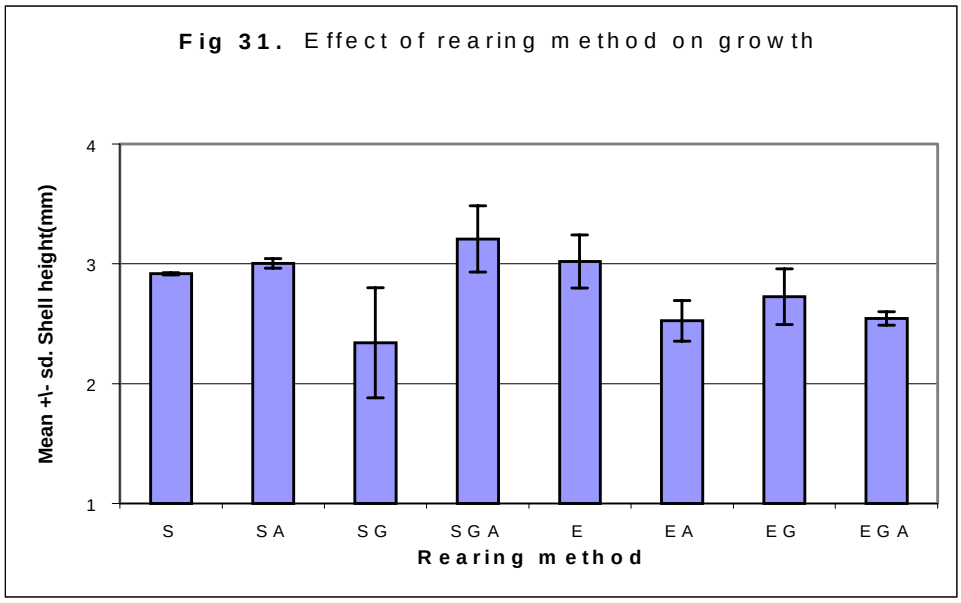
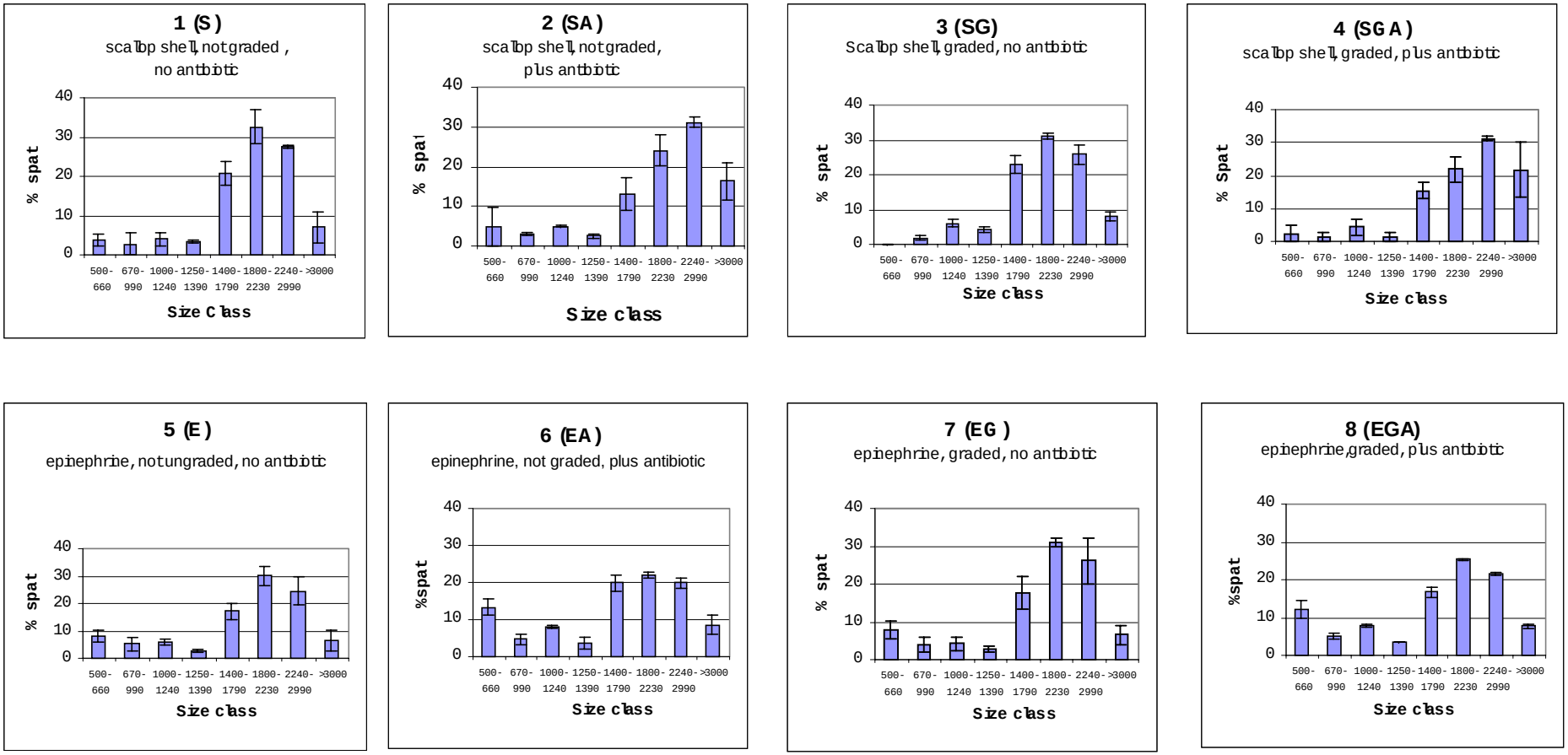


Fig 32. Effect of settlement technique, use of an antibiotic, and grading on the size frequency distribution of *S glomerata* juveniles



6.3.2 Spat mortality experiment 2

Investigation of effects of settlement technique, genetic selection and induced triploidy on the incidence and severity of the mass mortality disease of early *S. glomerata* spat

Background and aims
This experiment was run in conjunction with a large scale experiment (FRDC Project 93/15) to evaluate whether advantages conferred by selection for faster growth in *S. glomerata* (Nell et al., 1998) could be combined with and added to those conferred by cytochalasin C induced triploidy (Hand and Nell, 1999).

Methods
To meet the needs of the primary experiment aimed at assessing the single and combined effects of genetic selection and triploidy on the growth and survival and seasonal market condition of *S. glomerata*, the following four lines of larvae were separately reared in duplicate experimental scale (1 000L) vessels.

1. Non genetically selected diploid stock (code CTL-2N)
2. Non genetically selected triploid stock (code CTL-3N)
3. Genetically selected diploid stock (code L2-2N)
4. Genetically selected triploid stock (code L2-3N)

In addition to these, a much larger commercial batch of line 4 larvae i.e. genetically selected triploid stock (L2-3N) that initially yielded an estimated 12 million spat was separately reared in a 20 000L tank. Of the latter mass reared L2-3N larvae, three sub batches of competent larvae were stocked on separate screens and subjected to culchless settlement as single seed using epinephrine. Three sub batches of L2-3N larvae were also stocked onto each of three screens loaded with scallop shell culch to produce single seed. In all cases yields of single seed on each screen were similar.

Subsequently all six groups of spat were transferred to downweller nursery screens in the hatchery and thence to upweller screens in the field nursery system at Wanda Head, Port Stephens. All other rearing methods employed were identical to those described above for spat mortality experiment.

Results
Unlike experiment 1 most lines of spat sustained high rates of mortality. However manifestation of the disease, as indicated by cumulative mortality data was atypical. Unlike earlier episodes in which mortality occurred as one or a series of intense pulses over a few days, cumulative mortality data presented in Fig 33 and Fig 34 showed that mortality of spat on this occasion was progressive and protracted.

This atypical pattern of mortality occurred regardless of whether spat were settled on scallop shell or through culchless induction using epinephrine (Fig 30) and regardless of whether the stock were the progeny of 4th generation genetically selected parent stock or not (Fig 34).

These atypical results were however readily explained by pathology reports based on examination of samples of diseased spat sent to Dick Callinan (NSW Fisheries), Dr Judith Handlinger (Tas. DPIF), Dr Mike Hine (NIWA) and Dr Alex Hyatt (AAHL/CSIRO). All four pathologists concurred that the disease was clearly caused by invasive infections of a ciliate (*Uronema* sp.) (Appendices 6 & 7). Ciliates of the type had previously been identified by Dr Handlinger as responsible serious losses of pacific oyster (*Crassostrea gigas*) spat commonly experienced by commercial hatcheries in Tasmania over the past decade. Re-examination of archived samples of spat from previous mass mortality episodes as early as 1990, by Dick Callinan, Dr Handlinger and

Dr Hyatt however proved entirely negative for the involvement of *Uronema* sp. or any other type of ciliate. Unfortunately this involvement of *Uronema* sp. undermined any meaningful conclusions from this experiment on the likely benefits or otherwise of genetic selection, triploidy or method of settlement on bacterial mediated mass mortality disease of small juvenile *S. glomerata*.

Fig 33. Effect of settlment method on mortality of genetically selected triploid *S. glomerata* spat (L2-3N)

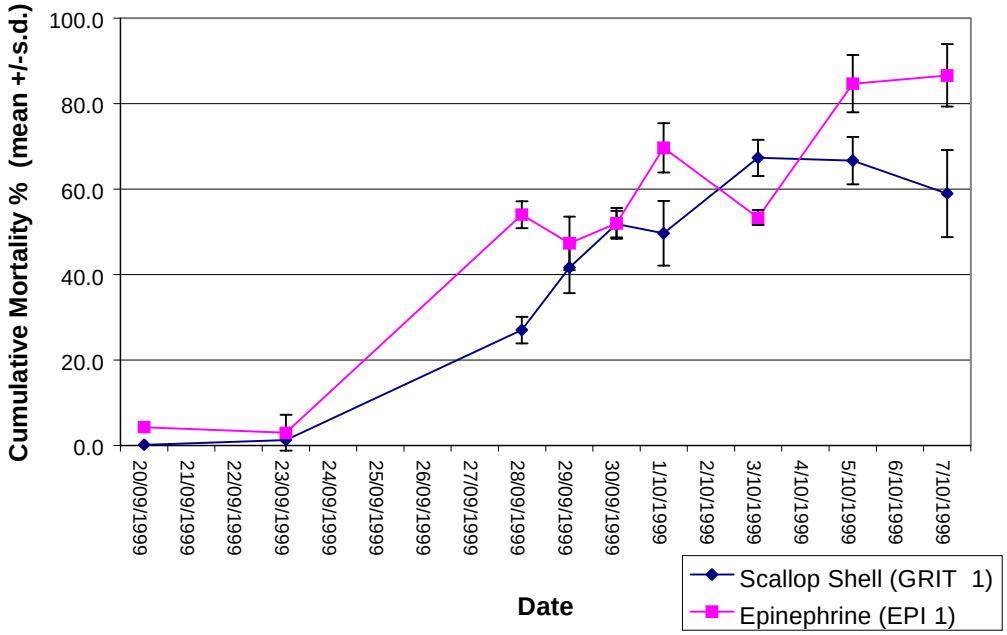
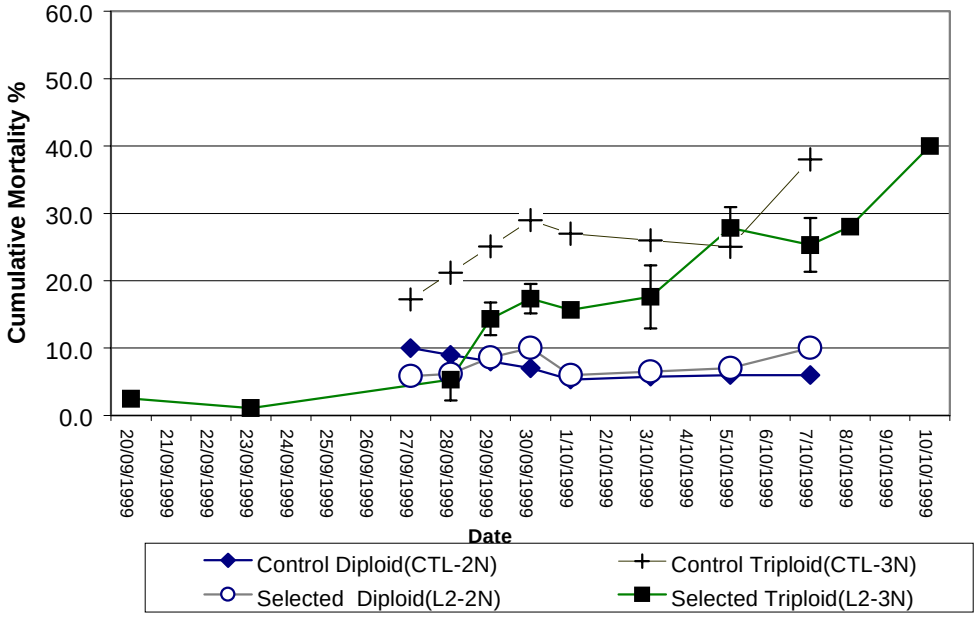


Fig 34. Effect of genetic selection and triploidy on mortality of *S. glomerata* spat (error bars when included =s.d.)



7.0 OVERALL CONCLUSIONS AND RECOMMENDATIONS

7.1 Mass Mortality Syndrome of Larval *S. glomerata*

- Results of epidemiological, histopathological and experimental investigations collectively point to the conclusion that this disease is caused by bacterial (*Vibrio* spp.) exotoxins that impact on the gut epithelium of young larvae and hence their ability to feed, grow and survive.
- The disease is highly seasonal and exacerbated through successive hatchery cycles. It is therefore recommended that future bivalve hatcheries comprise two or more fully independent modules that can be used and disinfected/dried out in alternation. It is also recommended that future hatchery production of *S. glomerata* in existing hatcheries be confined as much as is practicable to the lower risk season of July to December, that numbers of hatchery runs between consecutive cleaning, disinfection and 4 to 6 week dry-out operations be limited to one or two.
- Very encouraging experimental results have been attained when *S. glomerata* larvae were reared in seawater that had been sterilized then re-inoculated with selected non-pathogenic bacteria. Results showed that survival and growth of larvae over the first critical 8 days could be elevated from very low levels in control treatments typical of disease episodes to some of the highest rates ever experienced with *S glomerata* larvae over the past 15 years. It is therefore strongly recommended that this research being conducted in collaboration with the Dept of Cell Biology of the University of Technology, Sydney, be continued.
- It is recommended that future commercial scale hatchery protocols for *S. glomerata* be amended based on results of small-scale experimental trials on effects of temperature on fertilisation, incubation and hatchery rearing protocols. Of particular importance is a need to evaluate higher larval rearing temperatures than currently practised.

7.2 Mass Mortality Disease of Small (<2 mm) Juvenile *S. glomerata*

- Results of epidemiological, histopathological and experimental investigations collectively point to the conclusion that this disease is caused by high organic matter and *Vibrio* spp. bacterial loads associated with dense stocking rates in nursery screens and the use of ground scallop shell culch to produce single spat seed.
- Recommended strategies of overcoming this disease are to combine
    - cultchless production of single seed using epinephrine induction of metamorphosis or
    - develop commercially practical ways of setting oysters on flat surfaces that also enable creation of single seed once juveniles exceed 2 mm shell height
    - an avoidance of grading, counting and all other unnecessary handling of spat below 2 mm
    - development of low trauma high growth rate promoting rearing systems incorporating spat bubbler columns, controlled and optimised rearing temperatures and if necessary supplementary feeding with micro algae concentrate diets recently developed at PSFC (Heasman et al., 2000)
    - adapt and evaluate probiotic technology being developed to combat larval mortality diseases.

8.0 FURTHER DEVELOPMENTS

Mass Mortality Syndrome of larval S. glomerata
Continued research being conducted b NSW Fisheries in collaboration with the Dept of Cell Biology of the University of Technology, Sydney, to develop probiotic methods of preventing this disease is scheduled for completion in September 2002.

Mass mortality disease of small (<2 mm) juvenile S. glomerata
Development of low trauma and high growth rate promoting rearing systems incorporating spat bubbler columns and optimised rearing temperatures will be pursued over the same time frame as the larval disease R&D and will also incorporate probiotic technology as a means of combating the disease.

In addition, practical methods of propagating spat on two dimensional surfaces that can be fragmented in such a way as to generate single seed will be appraised.

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10.0 STAFF

NSW Fisheries Staff engaged on this project

Permanent staff
Dr Mike Heasman - Senior Research Scientist, Principal Investigator
Dr Richard Callinan - Departmental Veterinary Pathologist
John Diemar - Fisheries Technician Grade. 2/3
Lindsay Goard, - Fisheries Technician Grade. 2/3

Temporary staff
Celina Gartrell (1995) - Fisheries Technician Grade 1
Tanya Sushames (1996-1998) - Fisheries Technician Grade 1
Melissa Walker (1999-2000) - Fisheries Technician Grade 2

Collaborating UTS Staff

Dr Lewis Gibson -Principal Researcher –SPIRT/NSW Fisheries, Probiotic Project
Edward McGregor. BSc. (Hons) student (1999)
Cheok Tan, PhD student (2000)

11.0 APPENDICES

APPENDIX 1 Summary of hatchery data July 1988 to Dec 1999															
MS=mass selection; 3n=triploid; 2n=diploid; W=wild/control stock;? =unknown															
Date	**Operation**	**Larval Success (S/F)**	**Nursery / Spat Success (S/F)**	**Broods tock conditi oned (Y/N)**	**Tank Size (L)**	**Eggs (x10 ^6)**	**D-velige rs x10^6 (% yield)**	**Number to set system x10^6**	**Spat x10^6 (%yiel d)**	**Net spat from D veliger**	**Spat >2mm x10^6**	**Day larvae ceasedf feeding)**	**time since past SRO larval run / weeks**	**Days post set to spat mortality**	**Comments**
24-Jul-88	?	s	s		20000		61		13.98		11.3	5	new	nil	Excellent larval run. 11.3 million spat sold
5-Sep-88	?	s	f	?	20000		51.34		16.92		1.6		5	?	Large numbers moved to Vales Pt Nursery 14th October 88, 90% spat mortality
20-Mar-89	?	s	?	n	20000	?	34.4	?				5	18	?	Satisfactory larval run. Numbers to settlement unknown
24-Sep-89	?	s	s	y	20000/1 000 (D's)	?		2.88	0.96 (33%)				22	?	5% Spat mortality at 500um. No record after this observation. Successful run
6-Feb-90	MS	s	f		20000	?	55.6	8.36 (15%)			0	6	17	7 & 14	Larvae pale day 6. Severe mortality in upwellers 5/3/90-severe mortality in downwellers 12/3/90
31-Jan-91	MS	f		n	20000		96.8	0			0	5	44		Batch discarded. Larvae ceased feeding day 5 & did not resume. Strip spawned
14-Jan-92	MS	s	s	n	1000 x 12	96	60.8 (63%)	8.43 (13.9%)	6 (71%)	**9.9%**			50	nil?	Minor mortality 19 days post set. Good run

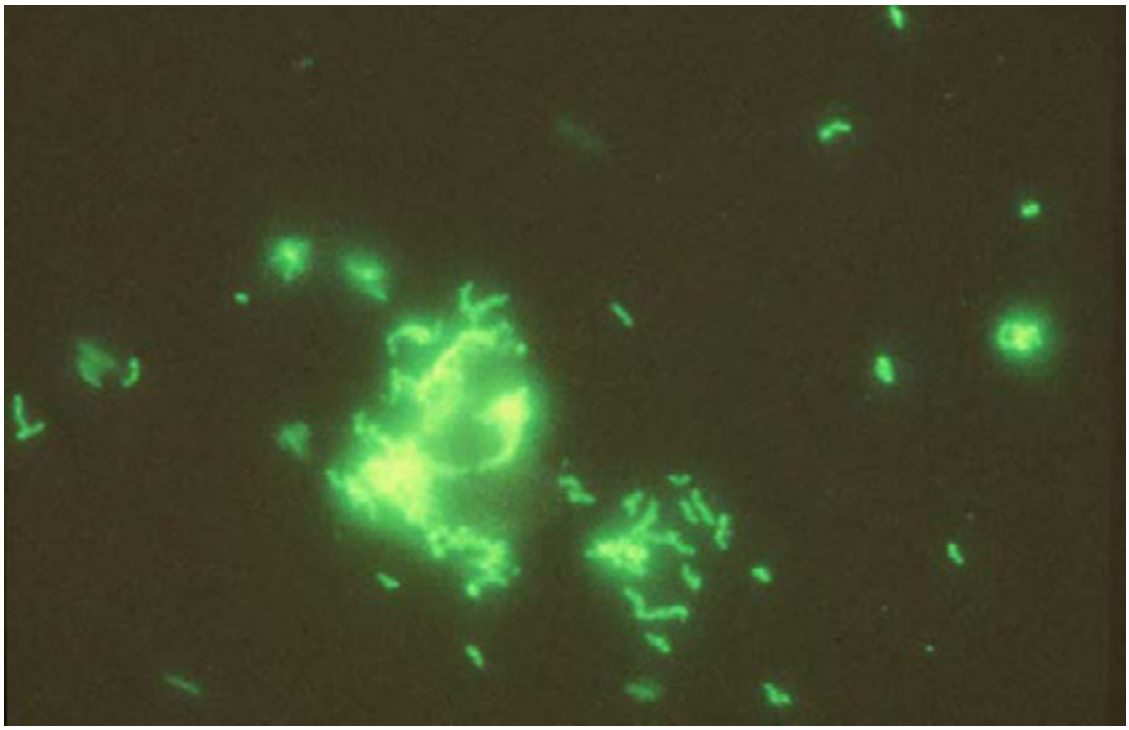
5-Jan-93	MS, Georges R. Wallis L. Hawkesbury R. Port S.	s	s	n	1000 x 12		70.59	4 (5.7%)	3.42 (86%)	**4.9%**			50	Nil ?	Overall set rate 86%. Poor larval growth and survival - satisfactory run.
11-Jan-94	MS	f		n	1000 x 16	48	40.9 (85%)	1.39 (3.4%)			0	10	52	?	Regarded as a failure. Guts light and dropout day 10
8-Feb-94	3N	s	f		20000		36.18	4.5 (12.4%)				11	3	?	60-70% spat mortality
	3N	s	f	n	20000		26.33	6 (22.8%)				4	3	10	Few pale larvae - but otherwise successful larval run. Mass mortality of spat. 10 days post set
22-Apr-94	MS	f	s	n	1000 x 6	96	60 (62.5)	1.37 (2.3)	0.065 (5%)	**1.2%**		6	9	nil	Regarded as a failure. Day 6, light guts. No spat mortality. Very few larvae put to set.
19-Dec-94	MS	s	f	n	1000	8		1.5			0		36	35	1.5 million pediveligers put to set for each line. Good larval run. Severe mortality 5 weeks post set (90 - 20 %)
23-Jun-95	Mass mortality	s	f		20000	130	13.21 (10%)	1.24 (9%)			0.22 (17%)		23	35	Strip spawned. Good larval result. Spat mortality 5 weeks post set, spat < 1800um almost all dead
12-Apr-96	Mass mortality	f		n	20000	100	36.4 (36.4 %)	0			0	5	39		Run aborted. Larvae failed to feed.
12-Apr-96	Mass mortality	f		n	20000	100	30.2 (30.2 %)	0			0	5	39		Run aborted. Larvae failed to feed.
25-Apr-96	Mass mortality	f		n	20000	60	57 (95%)	0			0	6	1		Run aborted. Larvae failed to feed. Poor water quality
25-Apr-96	Mass mortality	f		n	20000	60	54 (90%)	0			0	6	1		Run aborted. Larvae failed to feed. Poor water quality

31-May-96	Mass mortality Wild 2N	f	f	?	20000			0			0	2	4		No gut colour at all day 2. Run aborted
12-Nov-96	Antifoul exp.	s	?	?	10000	30	28 (93%)	4 (14%)					21	?	Good larval survival and food consumption
12-Dec-96	3N	s	?	?	20000	100	50.4 (50.4 %)	9.3 (18.5%)	2.4 (26%)		?	7	4	?	Small larvae not feeding well. Poor set on scallop shell. Algal blooms in tank.
17-Apr-97	3N	s	s	n	20000		64.13	1.86 (2.9%)	1.04 (56%)			6	16	?	Pale slow growing larvae early in run. Improvement observed after day 10
26-Nov-97	Wild 3N	s	f	n	20000		118.4	14.7 (12%)	5.5 (37%)		0.4 (7%)	2	28	11	Larvae appear light AM day 2. Improve afterwards. Good larval run. Massive Mortality of spat Jan 27th 1998
18-Sep-98	Wild 3N Manning R.	s	f	y	20000	95	72.24 (76%)	16.7 (23%)			0.745 (4.5%)	2	36	?	No spat grading or counting to try & avoid spat mortality. Spat suffered severe mortality over a 6 week period. Good numbers through larval cycle
13-Jan-99	MS 2N	f			20000	80	73.6 (91%)	0.1 (.001%)	0		0	2	20		Day 11, clear guts. Large size range. Large dropout of larvae day 12
13-Jan-99	MS 3N	f			1000	10	4.6 (46%)	0	0		0	6	20		Larval dropout and cessation of feeding day 6. Run aborted day 12
25-Feb-99	W 2N	f			1000	10	2.3 (23%)	0	0		0	5	new		Light guts. Larvae failed to feed. Tomaree site used. Run aborted.
26-Feb-99	MS 2N	f			250 FT	100	91.3 (91.3 %)	0	0		0	4	new		Light larvae. Failed run. Velum deformities - Tomaree. Run aborted
18-Aug-99	W 2N	s	f	y	1000	8	6.9 (86.2 %)	0.53 (7.7%)			0.054 (10%)	3	23	?	Slight paling of guts day 3. Successful larval run.

20-Aug-99	MS 3N	s	f	y	20000	160	108.1 (67.6 %)	20.3 (18.7%)	?		?	4	23	14	Spat mortality 28/9/99 - 2 weeks post settlement. Continued until December 99 caused by Uronema ciliates
20-Aug-99	MS 2N	s	f	y	1000	10	4.3 (43%)	0.58 (13.5%)	0.146 (25%)	**3.40%**		4	23	14	Larval run successful. Guts pale day 4. Mass mortality of spat 2 weeks post settlement.
17-Nov-99	Ciliate Mortality Trial MS 2N	s	f	n	20000	60	45.7 (76%)	5.61 (12%)	?		?	2	12		Good larval run. Settled spat numbers unknown.
Dec-99	MS FRI	s			4000										Mass Mortality of spat in Upwellers at Sans Souci

APPENDIX 2 SPAT PRODUCTION DATA					
Year	**Month**	**F**	**S**	**Days**	
1988	*July*		S		20000
1988	*Sept*	F		?	20000
1989	*Mar*	F		?	20000
1989	*Sept*		S?		1000?
1990	*Feb*	F		7 & 14	
1992	*Jan*		S		1000
1993	*Jan*		S		1000
1994	*Jan*				1000
1994	*Feb*	F		10	20000
9194	*April*		S		1000
1994	*Dec*	F		35	1000
1995	*Dec*	F		35	20000
1996	*Dec*	F			
1997	*April*		S		20000
1997	*Nov*	F		43	20 000
1998	*Sept*	F		?	20000
1999	*Aug*	F		18	20000
2000	*Jan*	F			
1999	*Nov*				

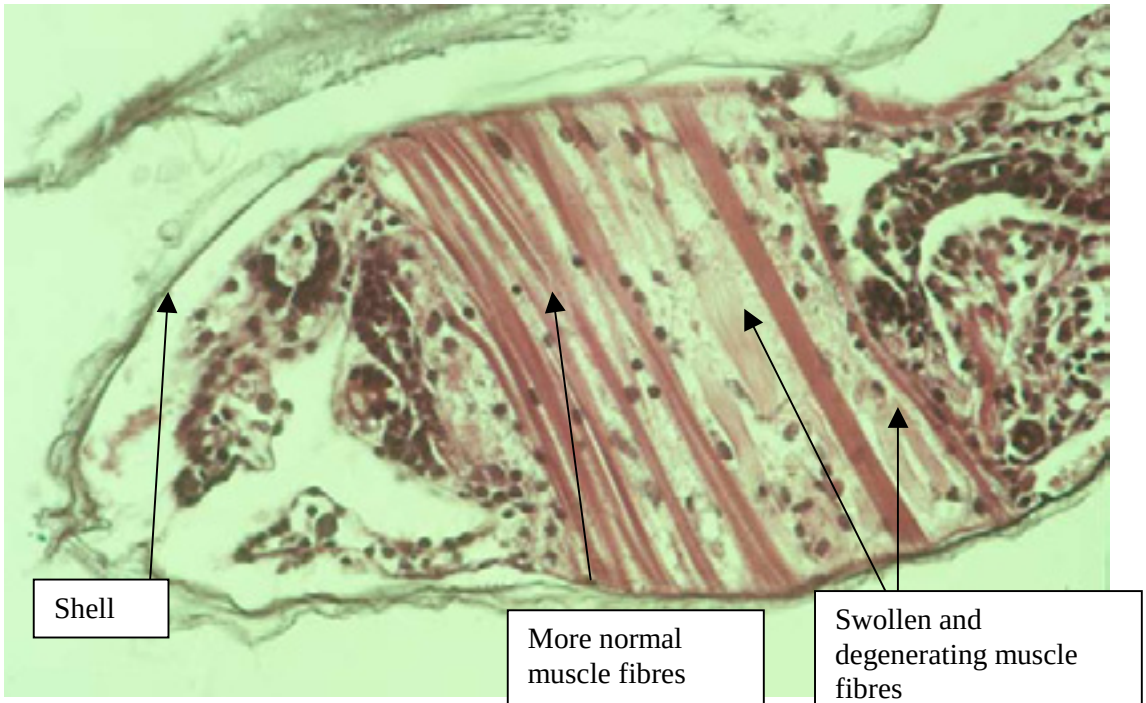
Appendix 3. Photomicrograph of a diseased juvenile *S. glomerata* showing *Vibrio alginolyticus* labelled using FITC immunofluorescence. Courtesy of Peter Hanna.



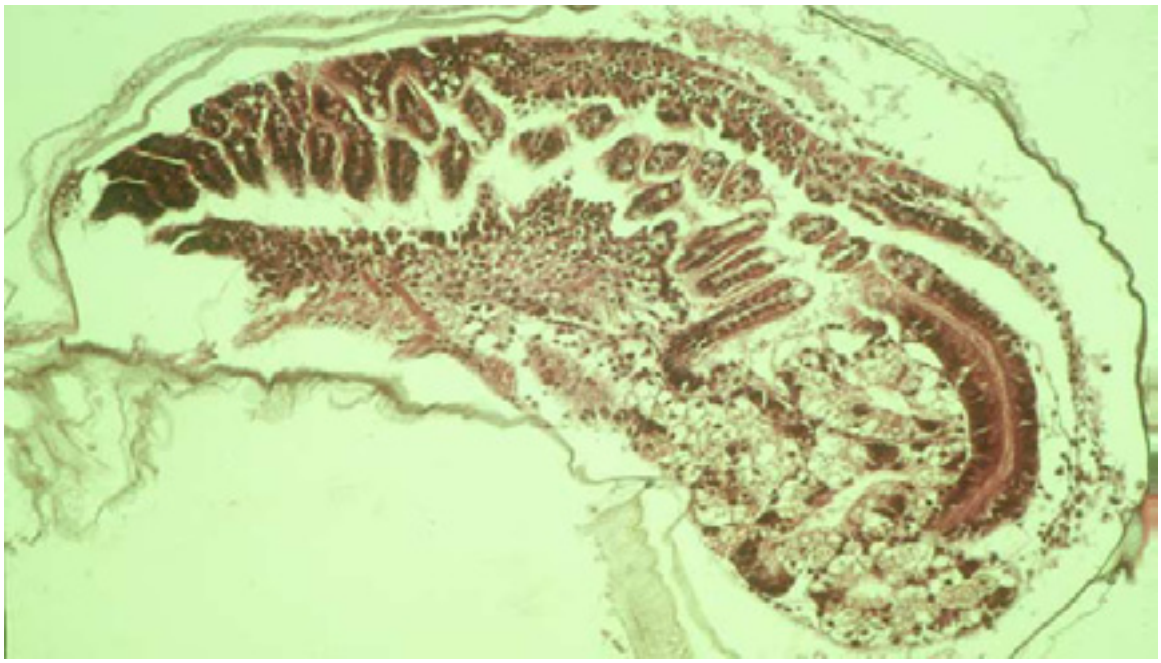
Appendix 4. Stained H & E section of healthy and diseased juvenile *S. glomerata*

Above: Relatively normal *S. glomerata* juvenile in cross section (Courtesy of Dick Callinan)

Below: Diseased gaping spat exhibiting degeneration of the adductor muscle fibres (Courtesy of Dick Callinan)



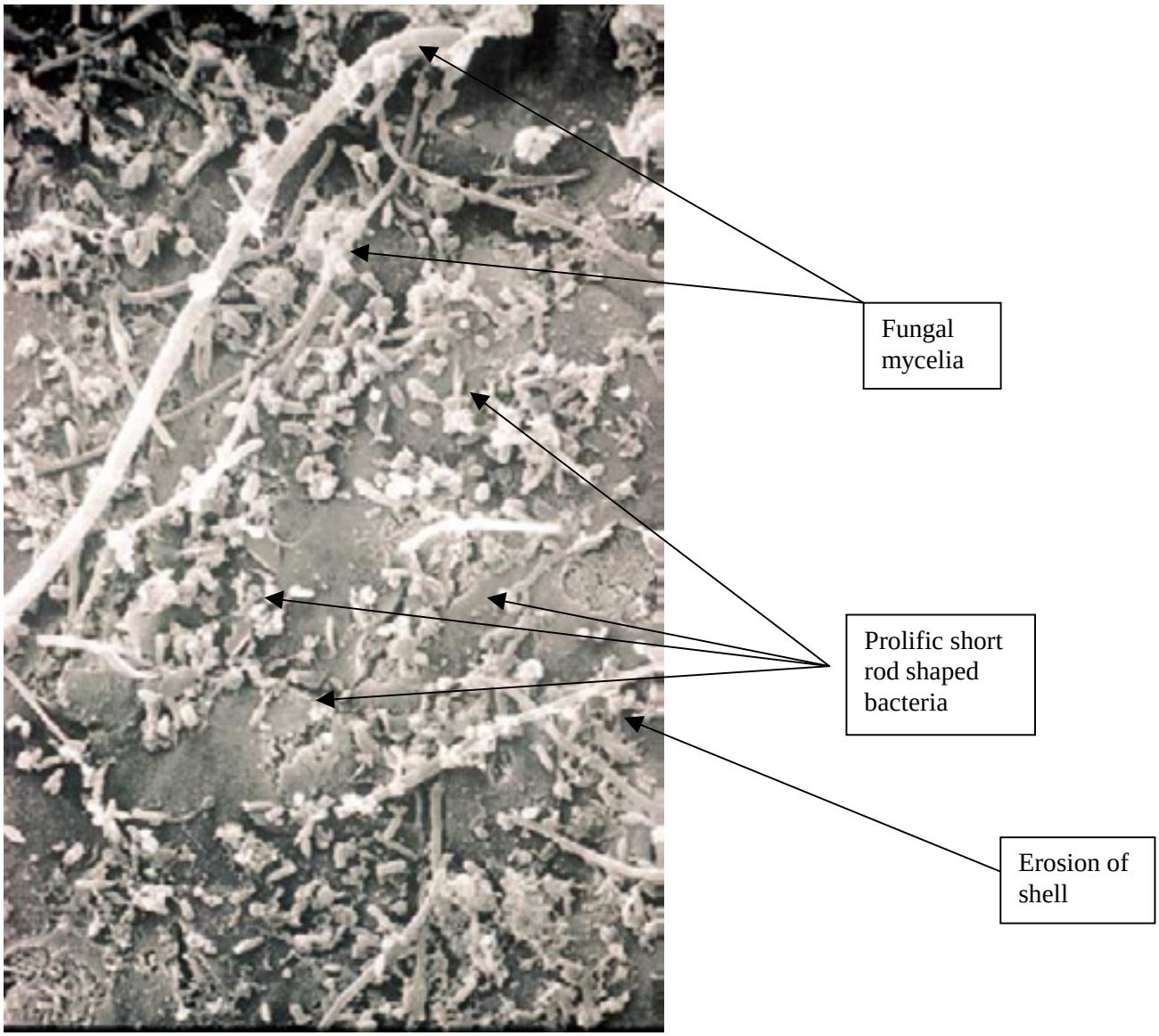
Appendix 4 Continued- Diseased juveni



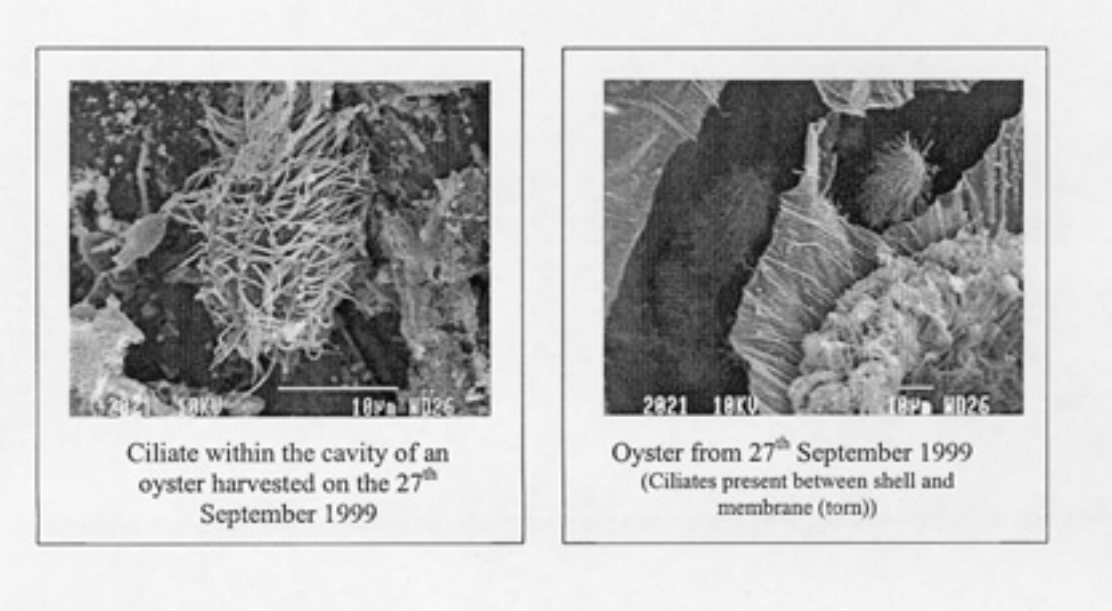
Note massive numbers of inflammatory cells (densely packed with nuclei) in the connective tissue

Digestive gland

Gut



Appendix 5. Electron photomicrograph of diseased juvenile *S. glomerata* showing massive numbers of rod-shaped bacteria and fungal strands. Note erosion of shell. Courtesy of Alex Hyatt. (AAHL/CSIRO)



Appendices 6 and 7 – Electron photomicrographs of invasive ciliates (*Uronema* sp.) in juvenile *S. glomerata* . Courtesy of Alex Hyatt.(AAHL/CSIRO)

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