TROPICAL AUSTRALIAN MICROALGAE AS LIVE FOOD FOR PEARL OYSTER (*Pinctada maxima)* SPAT

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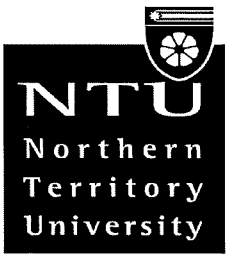

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Bibliography

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1. Pearl Oyster spat culture - Australia.
2. Pinctada maxima spat - Tropical Australianmicroalgae feed - Australia.
   I. Parry, D.L. (David L.) and Renaud S.R. (Sue R.).
   II. Fisheries Research and Development Corporation (Australia).
   III. Northern Territory University.
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OBJECTIVES:

1. To assess the suitability of Australian microalgae as live food source for young pearl oyster spat.

2. To prove that Australian tropical microalgae species are superior to the northern hemisphere species in the conditioning of spat of pearl oyster.

NON TECHNICAL SUMMARY

Currently the Australian pearl oyster industry is using live food consisting of mixed microalgae originating from overseas. These species include *Pavlova lutheri*, *Isochrysis galbana* (clone T. Iso), *Chaetoceros mulleri, Tetraselmis* sp. and *Cryptomonas sp.* Since these species were isolated from overseas, they may not perform optimally under Australian tropical conditions. A suite of tropical Australian microalgae are available from the Northern Territory University (NTU) and CSIRO Microalgae Collections, so this is an opportune time to test the above hypothesis.

Pearl oyster (*Pinctada maxima* (Jameson)) spat were fed on 10 species of tropical Australian micro-algae, *Chaetoceros* sp. (CS256), *Cryptomonas* sp. (CRFI01), *Fragilaria pinnata* (PS5), *Fragilaria pinnata* (GOC01), *Isochrysis* sp. (NT14), *Isochrysis* sp. (PS11), *Nephroselmis* sp. (GOC52), *Nitzschia paleacea* (NT7), *Tetraselmis* sp. (TEQL01), *Rhodosorus* sp. (CS249), either as unialgal, 100 % rations or in 50:50 combinations. The Tahitian *Isochrysis galbana* (strain T. Iso.) was included for comparison.

Spat fed on a 100 % ration of either CS256, CRFI01, T. Iso, PS 11, TEQL01, or NT7 for 4-6 weeks showed 700 - 2800 per cent and 360 - 460 per cent better growth and survival, respectively, than those of the unfed control. Spat fed CRFI01, PS11, CS256 or T.Iso had significantly higher organic weight, carbohydrate, lipid, protein and polyunsaturated fatty acid

(PUFA) contents at harvest than unfed controls. Spat fed CS256, PS11 or T.Iso. had higher amounts of the PUFA docosahexaenoic acid (DHA) than the unfed control, while spat fed CS256 or CRFI01 had higher amounts of eicosapentaenoic acid (EPA) than the unfed control. Spat fed 50:50 combinations of CRFI01 + T. Iso, PS11 + T. Iso, CS256 + T. Iso, CRFI01 + PS11, or CS256 + PS11 all showed significantly better growth than unfed control in terms of shell length, dry weight and organic weight. In terms of shell length and dry weight, the combination TIso/CS256 showed the best results. There were positive correlations between spat carbohydrate content and spat shell length ($r^2 = 0.717$), dry weight ($r^2 = 0.874$) and organic weight ($r^2 = 0.796$). There were also medium correlations between spat DHA and spat shell length ($r^2 = 0.595$). There were no correlations between spat physical parameters and microalgal ash, carbohydrate, lipid, protein or individual fatty acids.

The Australian microalgae *Chaetoceros* sp. (CS256), *Cryptomonas* sp. (CFRI01) and *Isochrysis* sp. (PS11) showed consistent performance comparable to that of T.Iso, and therefore could be used in the rearing of pearl oyster spat instead of the northern hemisphere *Isochrysis* (T. Iso). This means that it is possible to use these tropical Australian isolates in combination with overseas species in the rearing of Pearl Oyster spat. The reduction in use of overseas microalgal isolates could also reduce the possibility that the microalgae may enter the environment.

Further work needs to be done on

- feeding trials involving combinations of different Australian microalgae at different densities;
- feeding trials on industrial scales;
- growout performance of spat fed tropical Australian microalgae; and
- supplementary feeding trials at growout sites.

KEYWORDS: Tropical microalgae, pearl oysters, *Pinctada maxima*, aquaculture, nutrition

2. BACKGROUND

Since 1990, the Pearling Industry has routinely spawned the silver-lip (or gold-lip) pearl oyster, *Pinctada maxima* (Jameson), on a commercial basis (Gervis and Sims, 1992; O'Sullivan, 1994; Rose, 1994). The hatchery-spawned and -reared spat are then placed in the sea for the grow-out production phase. In 1996, the price for hatchery produced spat was high at approximately $US 0.1-0.14 per millimetre hinge length (Rose, R.A, unpublished data 1996, cited in Taylor et al., 1997a). Although hatchery methods for *P. maxima* are now well established (Rose and Baker, 1994), sporadic occurrence of high, unexplained mortalities experienced with spat recently entering ocean nursery facilities in the Northern Territory is still a problem (Rose, R.A. pers.comm.).

There are many hypotheses and complex reasons for the above mentioned mortalities. The high mortality of spat may be due to poor water quality or nutritional status of the spat, for example. Nutritionally, the high mortalities may be the result of two separated but interconnected events. Firstly, the high mortality may be due to the weak spat resulting from the union of poor quality gametes produced by the broodstock. Secondly, it may be due to the sudden switch from a hatchery diet (which consists of northern hemisphere phytoplankton) to a diet of local species present in the waters of the grow-out areas. In this work, we carried out preliminary investigation on the use of tropical Australian microalgae with the objective to test the second hypothesis.

The northern hemisphere microalgae that are currently being used as live food for pearl oysters and pearl oyster spat are: *Pavlova lutheri* and Tahitian *Isochrysis* sp. (T.Iso) for feeding *P. fucata* (Krishnan and Alagarswami, 1993; Numaguchi, 1994) and T.Iso, *Chaetoceros mulleri, Tetraselmis* and *Cryptomonas* for feeding *P. maxima* spat (Bellanger, 1995; Mills, 1997; Taylor et al., 1997b). A suite of tropical Australian microalgae are available from the NTU and CSIRO Microalgae Culture Collections, and it is an opportune time to test the above hypothesis. Moreover, the possible replacement of overseas microalgae species with Australian ones will simplify quarantine procedures for the disposal of the microalgae.

3. NEED

A bank of tropical microalgae isolated from the northern Australian waters by the microalgae group at the Northern Territory University (NTU) in Darwin and by the personnel at the CSIRO Marine laboratories in Hobart, Tasmania, is available at NTU and CSIRO. These microalgae species needed to be trialed to establish whether they could be used in the pre-conditioning of pearl oyster spat in the hatchery, so that the northern hemisphere phytoplankton could be replaced.

4. OBJECTIVES

a. To assess the suitability of Australian microalgae as live food source for young pearl oyster spat.

b. To prove that Australian tropical microalgae species are superior to the northern hemisphere species in the conditioning of spat of pearl oyster.

5. METHODS

5.1 Micro-algae

The following tropical Australian micro-algae were used in the feeding trials. They were chosen on the basis of size, ease of culturing and the taxonomic characteristics similar to the overseas species currently used by the industry (see background):

Cryptomonad:
Cryptomonas sp. (Isolated from Fitzroy Island, CRFI 01)

Diatoms:
Chaetoceros sp. (Isolated from Innisfail bay, CS 256)
Fragilaria pinnata Ehrenb.(Isolated from Port Steven, PS5)
Fragilaria pinnata Ehrenb.(Gulf of Carpentaria, GOC 01)

Green flagellates:
Nephroselmis sp. (Gulf of Carpentaria, GOC 52)
Tetraselmis sp. (MBPF Queensland, TEQL 01)

Rhodophyte;
Rhodosorus sp. (Isolated from Beaver Cay, CS 249)

Controls
Isochrysis galbana Parke (strain T. Iso.)
Unfed control.

Micro-algae were subject to a streak plate purifying procedure. Axenic conditions of the cultures was not achieved. However, the population of bacteria of the cultures has been kept to a low number as observed under phase contrast microscopy. The harmful bacteria, *Vibrio* sp., was not present in any cultures as tested using TCBS agar. Microalgae grown in 2 L batches until the end of the log phase, as determined by cell counts at intervals, were fed to the spat.

All microalgae were grown in 1.5 L sterile medium f in 2L flasks or 5L sterile medium f in 10L carboys at 27 °C, and 80-100 µmoles/photons m^{-2} s^{-1} under 14h:10h light/dark cycles. 0.5 - 1% CO_2 was bubbled in the cultures at a rate of 0.4 L min^{-1} as required to maintain pH of the cultures at 7.8- 8.0.

5.2 Experimental design of feeding trials

5.2.1 Feeding trials with single species of microalgae

The following methodology and experimental design were employed for the feeding trials:

Duration:	4-6 weeks
Location:	The Darwin Hatchery Project, Stokes Hill Wharf, Darwin
Water temperature:	Ambient (25 - 28 °C, minimum and maximum)
Water treatment:	The water used in feeding trials was pumped directly from the sea outside the hatchery to a settling reservoir. Once large particulates were settled out of the water, the water was then pumped through 10 μm sand filter, 5 μm cartridge filter and finally 1 μm cartridge filter. The 1μm filtered water was UV sterilised before use.
Water salinity:	35 +/- 1 $^{0}/_{00}$ as measured with a hand held refractometer.
Containers:	Spat were placed in 1.5L plastic containers containing 1L of above water, which was aerated with air stones. The containers were placed in a 10 tonne water bath to keep their water temperature constant.
Spat size:	6 - 15 mm
Number of spat per replicate:	15 - 20
Number of replicates:	3 - 6 replicates per treatment
Treatment:	10 new species of microalgae isolated from the Australian tropical waters (see species list above). These can be considered fixed factors which were chosen from a community of microalgae.
Controls:	Unfed and fed with *Isochrysis* Tahitian (T.Iso). Unfed control is used to identify new microalgae that cannot be used as feed, and T. Iso control is used to identify new microalgae that are superior or equivalent quality to those currently used as feed for Pearl Oyster spat.
Feeding history of spat:	Prior to experimentation, spat were fed a ration of T. Iso and *Chaetoceros mulleri*. After spat were placed in containers (see above), they were fed T.Iso for two days, then starved for two days before treatments were imposed. Unfed control ensures that the carry-over effect, if any, is accounted for.

At the beginning of each feeding trial, a sub population of spat of roughly equal shell length (range 0.610 - 0.770 mm, for all experiments, except the pilot experiment where spat range 1.520 -1.555 mm were used) was selected from a population of recently spawned broodstock. For each experiment, spat were selected and were randomly assigned to each replicate (15 spat per replicate for growth experiments, 20 spat per replicate for experiments involving biochemical analysis). Homogeneity amongst the replicates was statistically tested before each feeding trial. Replicated buckets were floated in a 10,000 L circulated water bath kept in an outdoor shade house. The position of the replicated buckets in the water bath was also selected by a randomised design. Spat in 3 replicate buckets were harvested at time zero for initial biochemical analysis (dry weight, ash weight, organic weight).

Algal density was adjusted to 100 000 cells per mL (see 4.2.2) at the beginning of each day and dead spat were removed. The water in each bucket was changed every 2 days. To monitor growth, spat shell length was measured weekly. The biochemical composition (carbohydrate, lipid, protein and fatty acid composition) of 3-5 replicate buckets of spat and 2 L of each microalgal feed, was analysed at the end of the experimental period.

5.2.2 Effect of microalgal concentration on spat growth rate

To establish the optimum feeding density of microalgae for pearl oyster spat, the species *Isochrysis* PS11 was fed to spat at three concentrations (0.3×10^5, 1×10^5 and 3×10^5 cells per mL). The controls were unfed spat and spat fed T.Iso. There were five replicates per treatment.

The following parameters were measured at the beginning and the end of the experiments: shell length, dry weight, ash weight and organic weight (ash-free dry weight).

Survival and specific growth rate (SGR) were calculated using the following equations.

$$\text{Survival } \% = (N_t - N_{to})/N_{to} \times 100$$

$$\text{SGR} = \frac{\ln SL_t - \ln SL_{t0}}{t - t_0} \times 100$$

Where SL_t is the shell length at time t, SL_{to} is the shell length at time t_o, N_t is the number of spat at time t and N_{to} is the number of spat at time t_o.

Specific growth rate was used so that results can be normalised between experiments.

5.2.3 Supplement feeding

The objective of this part of the study was to test the effect of combinations of promising species of Australian microalgae with or without *Isochrysis* (T.Iso.) on the growth and biochemistry of the spat. The following combinations (50:50 ratio based on organic weight of the algae) were used:

Cryptomonas CRFI01 + *Isochrysis* T. Iso
Isochrysis PS11 + *Isochrysis* T. Iso
Chaetoceros CS256 + *Isochrysis* T. Iso
Cryptomonas CRFI01 + *Isochrysis* PS11
Chaetoceros CS256 + *Isochrysis* PS11

Together with the above combinations, there were controls of unfed spat and spat fed T.Iso only. There were 8 replicates per treatment, with the duration of the experiment 28 days.

Parameters measured included shell length, dry weight, ash weight, ash-free dry weight, carbohydrate, lipid, protein and fatty acid composition. The first four parameters were measured at the beginning and the end of the experiment; the last four parameters, the end of the experiment only.

5.3 Biochemical analysis

For chemical analysis, harvested microalgae and spat were rinsed with 0.5 M ammonium formate, freeze-dried and stored at -75 °C prior to chemical analysis.

Duplicate samples of microalgal cells or spat were analysed for ash (inorganic matter) total carbohydrate, total lipid, total protein, and fatty acid composition. Total carbohydrates were determined by the colorimetric method of Dubois et al., (1956) and total lipid was analysed gravimetrically after extraction with chloroform-methanol (2:1) by the method of Bligh and Dyer (1959). Total inorganic matter (ash) was determined gravimetrically after heating at 550 °C. Total nitrogen content was determined by Flow Injection Analysis after Kjeldahl digestion and total protein calculated from total nitrogen (%) x 6.25. (Renaud et al., 1994).

Lipids extracted from microalgae and spat were saponified then acidified, the fatty acids extracted into hexane and then converted to fatty acid methyl esters (FAME) by digestion with 14% BF_3-methanol complex. The lipids from microalgal species were trans-methylated directly. FAME samples were reduced to 1 mL under a stream of nitrogen and analysed immediately or stored at -75 °C. Fatty acid compositions were determined using gas chromatography with a BP225, polar capillary column (50% cyanopropyl 50% phenyldimethylsiloxane, 25m x 0.2 mm id) and GC-MS using a non-polar column (DB-5MS, 30 m x 0.25 mm id) (Renaud et al., 1994). The shorthand notation used in fatty acid nomenclature is L:Bn-x where L = chain length, B = number of double bonds and (n-x) = position of the ultimate double bond from the terminal methyl group.

5.4 Statistical analysis

Significance at 0.05 level between the results were tested by ANOVA (SuperANOVA), followed by paired comparisons using Scheffe's or by Student-Newman-Kuels (S-N-K) test. Equality of variance was checked using Barlett's test. Correlation analyses of spat growth parameters (shell length, dry weight, organic weight) and biochemical composition of both microalgal food species and spat (ash, carbohydrate, lipid, protein, individual fatty acids) were carried out.

6. RESULTS

6.1 Spat fed single species of microalgae

6.1.1 Effect of different algae food on growth and survival of pearl oyster spat

The results described here (Table 1) are the summary of four separate feeding trials, extending from January 1995 to July 1996. At the end of the experimental period (4-6 weeks), spat fed CRFI01, CS256 showed better specific growth rates than the northern hemisphere microalga T. Iso. PS 11 performed at about 95% of the T. Iso control, whilst TEQL01 and CS249 showed 47% and 40%, respectively, below the T. Iso. control (Table 1). Those fed NT7, PS5, NT14, GOC01 showed poorer specific growth rates than that of unfed control (Table 1). It is interesting to note that GOC52 showed the best specific growth rate of all microalgae used, but also had the highest mortality of 93.3%.

6.1.2 Effect of different algal diets on the biochemistry of pearl oyster spat

Spat fed microalgal species CRFI01, PS11, CS256 and T.Iso control had organic weights at harvest that were significantly higher than those of the unfed controls (Table 2). The shell weights (ash) at harvest of spat fed CRFI01, PS11 and T.Iso were also significantly higher than the control (Table 2).

Further biochemical analysis of spat found that animals fed the microalgal species CRFI01, PS11, CS256 and T.Iso control, had higher carbohydrate, lipid and protein contents at harvest than unfed controls (Table 3).

Spat fed CRFI01, PS11, CS256 or T.Iso also had significantly higher polyunsaturated fatty acid (PUFA) contents at harvest (33.7-36 % total fatty acids) than unfed controls (Table 4). Spat fed CS256, PS11 or T.Iso. had higher amounts of the PUFA docosahexaenoic acid (DHA) than the unfed control (Table 4), while spat fed CS256, CRFI01 or NT7 had higher amounts of eicosapentaenoic acid (EPA) than the unfed control (Table 4).

6.1.3 Effect of microalgal concentration on spat growth rate and proximate chemical composition

Under the experimental conditions stated in section 4.2.1, the optimum microalgal concentration with respect to specific growth rate of spat was 3×10^5 cells per mL. Spat fed T.Iso or PS11 at concentration 3×10^5 cells per mL were found to have a significantly higher specific growth rate than spat fed either 0.3×10^5 or 1×10^5 cells per mL (Table 5).

Spat fed PS11 at concentration 3×10^5 cells per mL had significantly lower carbohydrate and lipid contents than all other treatments. There were no significant differences in protein or ash contents.

Table 1: Specific growth rate (SGR) and survival of pearl oyster spat fed on different microalgae over a 4-6 weeks period. Figures in brackets = 1 standard deviation. * = SD within replicates of the one experiment. 3-5 replicates per experiment. Each replicate had 15 spat. ** = calculated on basis of mean of all replicates. **[1] = value during first 2 weeks only, all spat died after this period.

Treatment	**Mean SGR of spat (% increase /day)**	**Mortality (%)**	**No. of experiment**
Nephroselmis sp. (GOC52)	0.810**[1]	93.3 (0)*	1
Cryptomonas sp. (CRFI01)	0.734 (0.308)	9.39 (7.98)	3
Chaetoceros sp. (CS256)	0.710 (0.231)	22.07 (34.81)	3
Isochrysis Tahitian (T. Iso)	0.685 (0.181)	18.56 (25.64)	4
Isochrysis sp. (PS11)	0.650 (0.141)	0.67 (0.95)	2
Tetraselmis sp. (TEQL01)	0.322 (0.168)	26.4 (3.39)	2
Rhodosorus sp. (CS249)	0.273**	84.47 (16.76)*	1
Unfed control	0.189 (0.098)	56.3 (36.58)	3
Nitzschia paleacea (NT7)	0.126 (0.048)	1.34 (1.90)	2
Fragilaria pinnata (PS5)	0.113**	39.97 (29.06)*	1
Isochrysis sp. (NT14)	0.05**	17.34 (12.09)	1
Fragilaria pinnata (GOC01)	-0.377**	77.7 (10.19)*	1

Table 2: The dry weight, organic weight and ash weight of spat at harvest. Data given as mean of 3 replicates with 15 animals per replicate. Standard deviation in parenthesis.

Algal species	Code	Dry weight (mg)	Organic weight (mg)	Ash weight (mg)
Unfed control		10.0 (3.1)	0.4 (0.2)	9.4 (2.9)
Isochrysis sp.	T.Iso	19.4 (5.1)	1.8 (0.5) *	17.5 (4.6) *
Chryptomonas sp.	CRFI01	20.6 (8.3)	1.9 (0.8) *	18.7 (7.5) *
Isochrysis sp.	PS11	16.7 (6.3)	1.5 (0.6) *	15.2 (5.8) *
Chaetoceros sp.	CS256	14.3 (3.6)	1.4 (0.4) *	12.9 (3.2)
Nitzschia paleacea	NT7	11.0 (2.5)	0.9 (0.3)	10.1 (2.3)
Tetraselmis sp.	TEQL01	11.0 (0.5)	0.9 (1.0)	10.0 (5.0)
Isochrysis sp.	NT14	8.9 (2.4)	0.9 (0.3)	8.0 (2.2)

* Significantly higher than unfed control, at 95% level, but not significantly different from one another. ANOVA and Scheffe's test were used.

Table 3: Proximate biochemical composition of **(A)** tropical Australian microalgal feed species, Day 28 (n = 2), and **(B)** pearl oyster spat fed new microalgae (data given as mean; n = 3; pooled samples, each consisting of 60 spat).

Algal species	Code	Proximate chemical analysis (% dry wt)			
		Ash	Carbohydrate	Lipid	Protein
(A) Microalgal food species					
Isochrysis sp. control	T.Iso	10.6	10.9	25.3	35.8
Cryptomonas sp.	CRFI01	14.8	4.0	22.3	48.1
Isochrysis sp.	PS11	11.8	14.6	22.7	24.3
Chaetoceros sp.	CS256	20.9	6.1	16.6	40.1
Nitzschia paleacea	NT7	21.1	6.8	24.3	38.2
(B) Pearl oyster spat					
Unfed control		94.0	0.22	0.21	5.79
Isochrysis sp. control	T.Iso	90.2	0.41 *	0.42 *	7.23
Cryptomonas sp.	CRFI01	90.8	0.38 *	0.45 *	7.41 *
Isochrysis sp.	PS11	91.0	0.40 *	0.50 *	7.46 *
Chaetoceros sp.	CS256	90.2	0.37 *	0.36 *	7.00
Nitzschia paleacea	NT7	91.8	0.27	0.24	6.29

* Significantly higher than unfed control, at 95% level, but not significantly different from one another. ANOVA and Scheffe's test were used.

Table 4: Fatty acid composition (% total fatty acids) of pearl oyster *Pinctada maxima* spat fed tropical Australian microalgae for 28 days (mean; n=2; pooled samples of 50 spat). Average coefficients of variation: ± 9.0 % for FAME < 1 % total fatty acids; ± 5.0 % for FAME > 2 % total fatty acids.

Fatty acid	**Fatty acid composition of spat (% total fatty acids)**						
	Unfed	**Microalgae food species**					
		TISO	**CRFI01**	**PS11**	**CS256**	**NT7**	**NT14**
Saturated fatty acids (SFA)							
14:0	2.8	3.5	2.5	3.8	1.1	2.3	3.3
16:0	17.4	18.1	20.6 *	18.8	18.6	22.8 *	26.7*
17:0	2.9	1.3	0.7	1.7	0.6	1.3	1.5
18:0	11.6	9.1	9.3	8.4	9.6	12.7	8.5
20:0	6.8	5.3	6.1	5.1	5.9	7.5	4.9
Total SFA	**41.5**	**37.3**	**39.2**	**37.8**	**35.8**	**46.5**	**44.9**
Monounsaturated fatty acids (MUFA)							
16:1n-7	3.4	2.1	5.1	1.1	1.1	1.7	1.8
18:1n-9	4.6	6.2	2.6	6.8 *	3.5	4.5	5.9
18:1n-7	3.6	3.8	5.1	3.1	2.8	4.2	2.3
20:1	1.5	2.4	1.0	2.3	1.8	1.1	2.1
22:1	7.8	9.1	5.3	8.7	10.3 *	7.0	6.9
Total MFA	**20.9**	**23.6**	**19.1**	**22.0**	**19.5**	**18.5**	**19.0**
Polyunsaturated fatty acids (PUFA)							
16:2n-7	2.2	1.1	1.7	0.9	1.1	2.5	1.8
16:3	-	-	-	-	-	-	-
18:2n-6	1.9	0.8	1.1	2.1	0.5	1.7	2.5
18:3n-3	-	2.1	0.9	0.9	-	-	0.6
18:4n-3	1.4	4.9 *	0.9	4.4 *	2.6	-	1.8
18:5n-3	-	-	-	1.0	-	-	-
20:2	-	-	-	-	-	-	-
20:4n-6	6.8	4.5	7.4	4.2	3.6	6.1	4.4
20:5n-3	1.5	1.0	6.3 *	0.9	3.8 *	4.7 *	1.6
22:2	3.2	1.7	4.4	1.7	2.3	3.7	0.9
22:4	1.5	1.3	1.0	1.1	0.9	1.3	1.2
22:5	3.8	4.8 *	2.1	4.8 *	5.4 *	3.2	5.6 *
22:6n-3	8.4	11.3 *	7.0	10.7 *	10.8 *	7.8	7.1
Total PUFA	**30.7**	**34.7 ***	**32.8**	**33.7 ***	**36.0 ***	**31.0**	**28.3**

* = Significantly higher than the unfed control

\- = Less than 0.1 % total fatty acids

Table 5: Specific growth rate (SGR) and proximate chemical analysis of pearl oyster spat fed different concentrations of microalgae (mean, n = 3; standard deviation in brackets).

Species	Concentration (cells per mL)	SGR (%)	Proximate chemical analysis (% dry wt)			
			Ash	Carbohydrate	Lipid	Protein
TISO	0.3×10^5	0.77 (0.05)*	89.3	0.94	0.51	4.61
	1×10^5	1.17 (0.19)	89.7	0.72	0.45	4.49
	3×10^5	1.32 (0.45)	88.0	0.76	0.43	4.59
PS11	0.3×10^5	0.63 (0.22)*	90.0	0.80	0.51	4.98
	1×10^5	1.13 (0.42)	89.7	0.81	0.56	6.96
	3×10^5	1.73 (0.12)	87.9	0.66 *	0.3 *	5.57
Unfed control: All died						

* = significantly lower than other treatments. ANOVA and Scheffe's test were used.

6.2 Spat fed a combination of two microalgae species (supplementary feeding)

6.2.1 Effect of supplementary feeding on mortality

The spat showed very little mortality over the 28 days of experimentation. The unfed control showed the highest mortality (Mean = 8%, Standard deviation (SD) = 4%). The three treatments TIso, TIso/PS11, and TIso/CRFI01 showed no mortality, whilst the next three treatments TIso/CS256, PS11/CRFI01, and PS11/CS256 had a mean of 1% mortality (SD = 2%) each. These mortality figures are in contrast with those in the experiment conducted in 1996 when there was greater than 50% mortality for all treatments. The difference in mortality may be due to the difference in water quality of the two seasons attributed to the cyclonic activities in 1996 season.

6.2.2 Effects of treatments on shell length, dry weight and organic weight (ash-free dry weight, AFDW) of spat

Table 6 shows the results of supplementary feeding experiments. As compared to the shell length at the beginning of the experiment, unfed control did not show any growth while all other treatments did. Dry weight and organic weight showed the same trend. In terms of shell length and dry weight, the combination TIso/CS256 showed the best results (significance at 5% level).

In terms of organic weight, the three treatments TIso, TIso/CRFI01 and TIso/CS256 were the best compared to all other treatments. However, there is no significant difference in organic weight between the four treatments TIso, TIso/CRFI01, TIso/CS256 and

Table 6: Effects of supplementary feeding on shell length, dry weight and organic weight of spat. Data given as mean, with standard deviation in parenthesis; n = 3 replicates with 15 animals per replicate.

Treatment	Shell length (cm)		Dry weight (mg)		Organic weight (mg)	
Unfed control	0.731	(0.049)	8	(1)	0.6	(0.2)
TIso	0.942	(0.072)	17	(3)	1.8	(0.5)
TIso/PS11	0.928	(0.086)	15	(3)	1.6	(0.5)
TIso/CRFI01	0.941	(0.093)	17	(4)	1.8	(0.4)
TIso/CS256	0.975	(0.079)	18	(3)	1.8	(0.4)
PS11/CRFI01	0.920	(0.066)	15	(3)	1.6	(0.4)
PS11/CS256	0.915	(0.085)	16	(6)	1.7	(0.4)

Table 7: Proximate biochemical composition of **(A)** tropical Australian microalgal feed species (mean, Day 0 and Day 28, n=3), and **(B)** pearl oyster *Pinctada maxima* spat fed tropical Australian microalgae for 28 days (mean, n=3, pooled samples of 50 spat). Average coefficients of variation: ash ± 7.8 %; carbohydrate ± 8.0 %; lipid ± 7.5 %; protein ± 7.5%.

	Proximate analysis (% dry weight)			
	Ash	**Carbohydrate**	**Lipid**	**Protein**
(A) Microalgae food species				
TISO	11.3	15.2	27.9	44.2
TISO + PS11	13.1	12.8	26.9	43.0
TISO + CRFI01	13.9	10.2	22.1	54.2
TISO + CS256	13.6	13.1	24.5	43.7
PS11 + CRFI01	15.9	9.2	22.2	50.2
PS11 + CS256	15.6	12.1	24.6	39.7
(B) Pearl oyster spat				
Unfed control	92.4	0.28	0.89	6.5
TISO	88.4	0.44	0.77	7.7
TISO+ PS11	89.4	0.39	0.79	8.3
TISO + CRFI01	89.3	0.52*	0.56*	9.1
TISO + CS256	90.3	0.45	0.57*	8.4
PS11+ CRFI01	88.2	0.47*	0.55*	8.9
PS11 + CS256	87.6*	0.45	0.56*	9.0

* Significantly higher than unfed control, at 95% level, but not significantly different from one another. ANOVA and Scheffe's test were used.

PS11/CS256. The ranking of the supplement feeding results in terms of organic weight is as follows:

TIso = TIso/CRFI01 = TIso/CS256 = PS11/CS256 > PS11/CRFI01 = TIso/PS11 > unfed control

Based on shell length, dry weight and organic weight, it would appear that TIso/CS256 may have the best potential as a live feed for pearl oyster spat.

6.2.3 Effect of different combined algal diets on the biochemistry of pearl oyster spat

Spat fed the combined microalgal species TIso/CRFI01 or PS11/ CRFI01 had significantly higher carbohydrate and lipid contents at harvest than unfed controls (Table 7). Significantly higher lipid content was also found in spat fed TIso/CS256 or PS1/CS256 (Table 7). There was no significant difference in protein contents of all treatments.

Spat fed combinations TIso/PS11, TIso/CRFI01, TIso/CS256 or PS11/CRFI01 had significantly higher percentages of polyunsaturated fatty acid (PUFA) at harvest (33.8-38.6 % total fatty acids), than the unfed control (Table 8). Spat fed the same four combinations or PS11/CS256 had significantly higher percentages of the PUFA docosahexaenoic acid (DHA) than the unfed control (Table 8). However, it is important to note that the absolute amount of fatty acid in the unfed control was very low.

6.2.4 Correlation between spat parameters, spat biochemistry or microalgal biochemistry

In feeding trials with single species of microalgae, there were positive correlations between spat carbohydrate content and spat shell length (r^2 = 0.717), dry weight (r^2 = 0.874) and organic weight (r^2 = 0.796). There were also medium correlations between spat DHA and spat shell length (r^2 = 0.595). In experiments investigating the effect of different combined algal diets, there were no correlations between spat physical parameters and microalgal ash, carbohydrate, lipid, protein (Table 7) or individual fatty acids (Table 9).

7. DISCUSSION

The Australian Pearl Oyster Industry has been very successful in the spawning of broodstock and the rearing of larvae and spat in hatcheries. It has been able to meet the demand of farmers for spat used in the growout phase. Currently, the industry is using live food consisting of mixed microalgae originating from overseas. This work is a preliminary investigation of the possible use of Australian tropical microalgae as a replacement for overseas species. The significance of the work is two fold. It may provide a better diet resulting in better growth and survival of spat; and if overseas microalgae species are replaced by Australian species, the quarantine procedures could be simpler.

The response of Pearl Oyster spat to different treatments shows that the pretreatment period for the experiments was adequate, and the 4-6 weeks duration was similar to other published work (Mills 1997). The positive specific growth rates and the high standard deviation of the mortality of unfed control (Table 1) could be due to the variation of the carry-over effect of the prehistory of the spat. The density of spat in containers was adequate as shown by the

ratio of shell height (SH) to shell length (SL) during growth (0.69-0.75). Taylor et al. (1997) reported abnormal growth in *P. maxima* with SH:SL of 0.9-0.93.

The results show clearly that Australian tropical microalgae have mixed quality as far as usage as live feed for Pearl Oyster spat is concerned. *Cryptomonas* sp. (CRFI01), *Chaetoceros* sp. (CS256) and *Isochrysis* sp. (PS11) have the potential of being able to replace the northern hemisphere Isochrysis sp. (T. Iso) in the rearing of Pearl Oyster spat. Specific growth rates and mortality rates of Pearl Oyster spat reported here for the above microalgae were comparable to that reported by Mills (1997) who fed spat with T. Iso and *Chaetoceros muelleri*. However, *Nitzschia paleacea* (NT7), *Fragilaria pinnata* (PS5 & GOC51) and *Isochrysis* sp. (NT14), and to certain extent *Rhodosorus* sp. (CS249), were not suitable as a source of live food for Pearl Oyster spat.

Although the above results are preliminary, it is interesting to note that CRFI01, CS256 and GOC52 are better food for *Artemia salina* than T. Iso, and that NT14 performs worse than unfed control (Luong-Van Thinh et al. 1999). One of the reasons for the poor performance of NT14 may be due to volatile chemical compounds secreted by the microalge, such as an unidentified long-chain alkanone, which was detected by gas chromatography but not further characterized (Luong-Van Thinh et al. 1999).

It is clear from these results that spat fed Australian tropical microalgae CRFI01 and CS256 had higher concentration of 20:5n-3 than T. Iso and unfed control. Supplementary feeding experiments involving mixed diets of microalgae showed that spat constantly had higher concentrations of 22:6n-3 than those fed T. Iso alone and unfed control. These polyunsaturated fatty acids are essential to the growth and development of many aquaculture animals (Brown et al. 1989), and they could influence survival in the growout situation. On the other hand, gross energy and digestibility (see below) may play an important role. This hypothesis may be tested in the next phase of the project. It is worth mentioning here that gross energy content of dietary ingredients does not account for the ability of an organism to digest that energy (Fleming et al. 1995).

There were no clear correlations between the gross chemical composition of mixed feed of microalgae and growth of spat. The same results were found in supplementary feeding of juvenile Pacific Oysters, *Crassostrea gigas* (Brown & McCausland 1999). It is possible that mixed diet induces digestive enzymes formation in the gut of spat. This prepares spat to digest other live food present in the growout environment, which influences survival of the spat. Mixed diet also stimulates the formation of a balanced gut flora of spat, and enhances the digestive physiological ontological changes. This may lead to increased filtering rate, digestibility and physical quality of the spat, resulting in higher growth and survival rates.

In conclusion, this preliminary work shows the potential of several Australian tropical microalgae in the rearing of Pearl Oyster spat. Extensive further work is needed to confirm that the Australian microalgae are superior to the existing ones used by the industry.

8. BENEFITS

It is clear from the above results that the Australian microalgae *Chaetoceros* sp. (CS256), *Cryptomonas* sp. (CFRI01) and *Isochrysis* sp. (PS11) showed consistent performance and therefore could be used in the rearing of pearl oyster spat instead of the northern hemisphere *Isochrysis* (T. Iso). These results could be further refined by future research and implementation. The use of Australian microalgae would benefit the industry in terms of possible environmental problems created by imported microalgae species.

9. FURTHER DEVELOPMENT

It is clear from the above results that further work on the Australian microalgae *Chaetoceros* CS256 and *Cryptomonas* CRFI01 is warranted. Further supplementary feeding experiments can be conducted with these two Australian microalgae used as the base to which other microalgae can be combined. Different microalgal combinations (e.g., other than the 50:50 ratio used in this experiment) can also be used. Additional parameters should be included such as filtering rate, digestibility, and quality of the shell (form, shape, toughness), and the nutritive value (especially the energy content) of individual and mixed microalga feed (van Barneveld 1995) . Larger scale feeding trials should then be carried out.

Monitoring of spat growth and survival in the growout phase should be planned to confirm the performance of spat reared on Australian microalgae. Supplementary feeding of spat under grow out conditions should also be planned.

10. CONCLUSION

The Australian microalgae *Chaetoceros* sp. (CS256), *Cryptomonas* sp. (CFRI01) and *Isochrysis* sp. (PS11) showed consistent performance comparable to that of T.Iso, and therefore could be used in the rearing of pearl oyster spat instead of the northern hemisphere *Isochrysis* (T. Iso). On the basis of the growth of spat, the Australian microalgal species were superior to T. Iso. However, on the basis of nutrition, further work is required to form a firm conclusion. The outcomes above are consistent with the objectives of the project, which state

1. To assess the suitability of Australian microalgae as live food source for young pearl oyster spat.

2. To prove that Australian tropical microalgae species are superior to the northern hemisphere species in the conditioning of spat of pearl oyster.

Table 8: Fatty acid composition (% total fatty acids) of pearl oyster *Pinctada maxima* spat fed tropical Australian microalgae for 28 days (mean; n=2; pooled samples of 50 spat). Average coefficients of variation: ± 9.0 % for FAME < 1 % total fatty acids; ± 5.0 % for FAME > 2 % total fatty acids.

Fatty acid	**Fatty acid composition of spat (% total fatty acids)**						
	Microalgal food species						
	Unfed	**TISO**	**TISO/PS11**	**TISO/CRFI01**	**TISO/CS256**	**PS11/CRFI01**	**PS11/CS256**
Saturated fatty acids (SFA)							
14:0	1.6	4.9 *	6.1 *	3.0	3.0	2.5	2.5
16:0	16.7	17.2	15.1	16.1	15.8	15.6	16.0
17:0	2.3	2.1	1.8	2.6	2.4	2.4	2.3
18:0	10.2	7.4	6.1	6.8	6.1	6.5	6.1
20:0	5.0	3.5	4.3	5.1	5.0	4.9	5.3
Total SFA	**35.8**	**35.1**	**33.4**	**33.6**	**32.3**	**31.9**	**32.5**
Monounsaturated fatty acids (MUFA)							
16:1n-7	2.6	3.5	2.1	2.6	5.8	2.5	5.6
18:1n-9	5.9	8.0 *	9.5 *	4.9	4.8	5.2	5.3
18:1n-7	2.5	3.3	3.2	2.5	3.1	2.2	3.0
20:1	4.4	2.7	3.6	2.9	2.5	3.1	2.6
22:1	9.5	8.5	8.3	8.1	6.7	8.7	7.3
Total MFA	**24.9**	**26.0**	**26.7**	**21.0**	**22.9**	**21.7**	**23.8**
Polyunsaturated fatty acids (PUFA)							
16:2n-7	1.1	2.6	2.0	3.9	3.7	5.4	4.0
16:3	-	1.9	1.7	4.3	3.2	2.0	2.8
18:2n-6	1.9	3.0	3.1	2.2	2.1	1.8	1.9
18:3n-3	0.1	1.5	1.5	3.5	0.2	3.6	0.3
18:4n-3	0.1	5.3 *	6.4 *	4.5	2.7	3.8	1.8
18:5n-3	-	-	0.9	0.8	1.0	0.2	0.2
20:2	2.1	1.2	1.1	0.1	1.1	1.0	1.1
20:4n-6	5.0	3.3	3.3	3.5	4.1	3.4	4.0
20:5n-3	3.1	0.9	0.6	1.6	2.1	1.9	2.4
22:2	3.2	0.1	1.2	1.5	2.4	1.2	3.0
22:4	1.4	1.3	0.9	0.3	0.3	1.4	0.6
22:5	3.4	3.5	3.7	4.4	3.2	4.3	2.9
22:6n-3	6.4	7.2	8.2 *	8.0 *	7.7 *	8.3 *	7.8 *
Total PUFA	**27.8**	**31.8**	**34.6***	**38.6***	**33.8***	**38.3***	**32.8**

* = significantly higher than the unfed control

Other abbreviations as in Table 5

Table 9: Fatty acid composition (% total fatty acids) of mixed Australian tropical microalgal species fed to pearl oyster *Pinctada maxima* spat (mean, Day 0 and Day 28, n = 4). Average coefficients of variation: ± 9.0 % for FAME < 1 % total fatty acids; ± 5.0 % for FAME > 2 % total fatty acids.

Fatty acid	**Fatty acid composition of microalgae (% total fatty acids)**					
	TISO	**TISO/PS11**	**TISO/CRFI01**	**TISO/CS256**	**PS11/CRFI01**	**PS11/CS256**
Saturated fatty acids (SFA)						
14:0	29.9	28.1	16.0	21.7	14.5	20.3
16:0	11.3	10.6	13.3	18.4	12.7	17.8
18:0	0.5	0.6	1.2	0.7	1.2	0.7
Total SFA	**41.7**	**39.3**	**30.5**	**40.8**	**28.4**	**38.8**
Monounsaturated fatty acids (MUFA)						
16:1n-7	5.8	5.3	4.4	20.7	3.9	20.2
18:1n-9	10.8	11.2	6.6	5.8	7.0	6.2
18:1n-7	0.7	1.2	1.0	0.7	1.5	1.2
20:1	-	-	-	-	-	-
Total MFA	**17.3**	**17.7**	**12.0**	**27.2**	**12.4**	**27.6**
Polyunsaturated fatty acids (PUFA)						
Polyunsaturated fatty acids (PUFA)						
18:2n-6	3.7	4.3	2.4	2.1	3.0	2.7
18:3n-3	4.2	3.7	12.7	2.1	12.8	2.1
18:4n-3	16.1	15.7	20.1	8.1	20.3	8.2
18:5n-3	4.6	4.8	4.5	4.5	5.2	5.1
20:4n-6	-	-	0.7	0.4	0.7	0.4
20:5n-3	0.1	0.2	3.6	2.1	3.7	2.2
22:6n-3	3.9	2.9	3.2	1.3	3.7	1.8
Total PUFA	**32.6**	**31.6**	**47.2**	**20.6**	**49.3**	**22.5**

Abbreviations as in Table 5

11. RECOMMENDATIONS

Based on the results of this work, it is recommended that pearl hatchery operators could use the Australian microalgae *Chaetoceros* sp. (CS256), *Cryptomonas* sp. (CFRI01) and *Isochrysis* sp. (PS11) to supplement T. Iso in the rearing of small spat in the range of 0.600 - 1.500 cm shell length.

12. REFERENCES

Bellanger, J., 1995. Effects of food density on the feeding physiology and resultant scope for growth of juvenile pearl oysters, *Pinctada maxima* (Jameson) and *P. albina* (Lamark). Honours Thesis, James Cook University, Queensland, Australia, 99pp.

Bligh, E.G., Dyer, W.J., 1959 A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911-917.

Brown, M.R., Jeffrey, S.W., Garland, C.D., 1989. Nutritional aspects of microalgae used in mariculture; a literature review. CSIRO Marine Laboratories Report 205, 44pp.

Brown, M.R., McCausland, M.A., 1999. Increased production of juvenile Pacific Oysters (*Crassostrea gigas*) through supplementary feeding. FRDC Project 94/83 Report, 159 pp.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for the determination of sugars and related substances. Analyt. Chem., 18: 350-356.

Fleming, A.E., van Barneveld, R.J., Hone, P.W., 1995 The development of artificial diets for abalone: a review and future directions. In: the use of agricultural grains and legumes in aquaculture feeds workshop, May 29-30, 1995. South Australian Research and Development Institute.

Gervis. M.H. and Sims, N.A., 1992. The biology and culture of pearl oysters (Bivalva:Pteriidae). ICLARM Stud. Rev. 21, ODA (Publ.), London, 49pp.

Krishnan, A. and Alagarswami, K., 1993. Effect of larval density and algal cell concentration on hatchery rearing and production of the Indian pearl oyster *Pinctada fucata* (Gould). In: Natarajan , P. and Jayaprakas, V. (eds.) Proceedings of the National Seminar on Aquaculture Development in India-Problems and Prospects, Trivandrum, India, 27-29 Nov. 1990, Kerala University, pp 123-130.

Luong-Van, Thinh, Renaud, S.R., Parry, D.L., 1999. Evaluation of recently isolated Australian tropical microalgae for the enrichment of the dietary value of brine shrimp, *Artemia nauplii*. Aquaculture, 170: 161-173.

Mills, D., 1997. Evaluation of histological cassettes as holding containers for individual spat, and a weekly handling protocol to assess growth of the silver-lip pearl oyster, *Pinctada maxima* (Jameson). J. Shellfish Res., 16 (2): 555-559.

Numaguchi, K., 1994. Studies on the feeding ecology and food environment of the Japanese pearl oyster, *Pinctada fucata* martensii. PhD Thesis, Nagasaki University.

O'Sullivan, D., 1994. Hatchery boost for Australia's pearl oysters. Fish Farm. Int., 21 (2):31-33.

Renaud, S.M., Parry, D.L., Thinh L-V., 1994. Microalgae for use in tropical aquaculture. I. Gross chemical and fatty acid compositions of twelve species of microalgae from the Northern Territory, Australia. J. appl. Phycol., 6: 337-345.

Rose, R.A., 1994. Development of pearl oyster hatcheries in Australia. Pearl World, Int. Pearl. J., 2(1): 6.

Rose, R.A. and Baker, S.B., 1994. Larval and spat culture of the Western Australia silver- or goldenlip pearl oyster, *Pincdata maxima* Jameson (Mollusca: Pteriidae). Aquaculture, 126: 35-50.

Taylor, J.J., Rose, R.A., Southgate, P.C., and Taylor, C.E., 1997a. Effects of stocking density on growth and survival of early juvenile silver-lip pearl oysters, *Pinctada maxima* (Jameson), held in suspended nursery culture. Aquaculture 153: 41-49.

Taylor, J.J., Southgate, P.C, Wing, M.S., and Rose, R.A., 1997b. Assessment of the nutrient value of five species of microalgae for spat of the silver-lip pearl oyster, *Pinctada maxima* (Mollusca: Pteriidae) (Jameson). Asian Fisheries Sci., 10: 1-8.

van Barneveld, R.J., 1995 Defining the nutritive value of feed ingredients - considerations for aquaculture. In: the use of agricultural grains and legumes in aquaculture feeds workshop, May 29-30 1995. South Australian Research and Development Institute.

Appendix 1

Intellectual property

No intellectual property can be placed on these microalgae since they are available at the NTU and CSIRO microalgal collections.

Appendix 2

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