

Chapter 4

Lectins as probes to evaluate the role of signaling molecules from the bacteria

4.1 Introduction

Bacterial biofilms have been indicated to be important in the settlement processes of planktonic larvae of most marine invertebrate groups (Crisp 1984; Mitchell 1984). The larvae are attracted towards specific chemicals in the microbial films that serve as cues for the settling larvae. Examples include spirorbid polychaetes (Knight-Jones 1951; Meadows and Williams 1963; Kirchman et al. 1982a), bryozoans (Mihm et al. 1981; Brancato and Woollacott 1982), ascidians (Szewzyk et al. 1991; Wieczorek and Todd 1997), echinoderms (Cameron and Hinegardner 1974; Johnson et al. 1991), phoronids (Herrmann 1975), sponges (Woollacott and Hadfield 1996), cnidarians (Müller et al. 1976; Hofmann et al. 1978; Neumann 1979), mollusks (Scheltema 1961; Weiner et al. 1985) and oysters (Weiner et al. 1985).

Barnacle cyprids, like most other larvae, also prefer to settle on substrata that possess a well-developed biofilm (Crisp 1984; Clare et al. 1992). A number of investigators have assessed the effects of pure cultures of bacteria isolated from biofilms on the larvae of choice in the laboratory (Maki et al. 1988,1992,1994; Holmström et al. 1992,1996; Avelin Mary et al. 1993; O'Connor and Richardson 1996,1998; Anil and Khandeparker 1998; Khandeparker et al. 2002). Earlier investigations have reported stimulation, inhibition or no effect of bacterial films on the attachment of barnacle cyprids (Crisp and Meadows 1962; Harris 1946; Visscher 1928; Tighe-Ford et al. 1970; Neal and Yule 1994a,b; Maki 1999). Olivier et al. (2000) also showed that the degree of microbial pre-colonization was the main parameter affecting the settlement of *B. amphitrite*. Recently a thraustochytrid protist, detected in

marine microbial films has been shown to induce the settlement of *B. amphitrite* (Raghukumar et al. 2000).

After attaching to a surface, bacteria not only reproduce but also exude mucous materials to form a layer of biofilm (Costerton et al. 1978,1995), known as exopolymers. These are composed of polysaccharides with variable amount of proteins. The extracellular polymeric substances (EPS) either contribute to the biofilm matrix (Cooksey 1992; Costerton et al. 1994) or are released by the microorganisms to the surrounding medium as planktonic or free EPS (Sutherland 1985; Beech 1990; Beech et al. 2000; Khandeparker and Bhosle 2001). The larval responses thus depend on the surface, which is changed by the extracellular molecules (Baier 1984) and the nature of the extracellular products. Maki et al. (1988) has reported the involvement of exopolymers produced by the bacteria in the attachment response of the larvae of *B. amphitrite*. Bacteria can also produce surface-bound and soluble chemical cues that either stimulate or inhibit larval settlement (Kirchman et al. 1982a; Maki et al. 1990,1992; Szewzyk et al. 1991). Bacterial extracellular products are also reported to be involved in the attachment of larvae of a spirorbid polychaete (Kirchman et al. 1982b) and an oyster (Weiner et al.1985).

Lectins, a class of naturally occurring proteins or glycoproteins exist in almost all living organisms and can recognize and bind carbohydrates specifically and noncovalently. The role of lectins in larval settlement has been investigated in several studies (Kirchman and Mitchell 1981,1983,1984;

Kirchman et al. 1982a,b; Maki and Mitchell 1985; Mitchell 1984; Mitchell and Kirchman 1984; Mitchell and Maki 1988). The settlement of a polychaete, *Janua brasiliensis* was also mediated by lectins on the larval surface that are proposed to recognize and bind to the bacterial polymer containing glucose (Kirchmann et al. 1982a). In case of *Crassostrea*, an oyster, neither the lectins nor monoclonal antibodies blocked the ability of *Alteromonas colwelliana* films to cue set the larvae (Weiner et al. 1989). No such studies are reported in case of barnacles to the best of our knowledge, although *Lens culinaris* agglutinin (LCA)-binding sugar chains of the settlement-inducing protein complex (SIPC) which induces gregarious settlement in this species have been suggested to be involved in the settlement of *B. amphitrite* (Matsumura et al. 1998). The present investigation was undertaken in order to identify the specific carbohydrate molecules from the bacterial film that are responsible for metamorphosis facilitation.

The bacteria, *P. aeruginosa*, *B. pumilus*, *C. freundii* isolated from the biofilms present on the shell surfaces of *B. amphitrite* were used in the present investigation. The primary strategy was to block the determinants of the bacterial films using lectins that would bind to specific carbohydrate moieties present in the extracellular polysaccharides on the bacterial surfaces. A comparison of the responses of the larvae to lectin-treated bacterial films and untreated bacterial films would unravel the possible mechanisms for the induction of larvae of *B. amphitrite* by bacterial films and the specific carbohydrate moiety of the polysaccharide involved in signaling the larvae.

4.2 Materials and methods

4.2.1 Preparation of adult extract (AE)

AE was prepared by following the method described earlier by Larman et al. (1982). Adult specimens of *B. amphitrite* were collected from the intertidal area of Dona Paula (15° 27.5'N, 73° 48'E). After being brought to the laboratory they were cleaned by brushing off the epibiotic growth on their shells using a nylon brush. The animals were then washed with deionised water. Approximately 100-g wet wt. of whole adults was crushed with a mortar and pestle using 100 ml of deionised water (RO pure). The supernatant of the crushed mixture was decanted, centrifuged (12,000 x g for 5 min), and thereafter boiled for 10 min in a boiling water bath. The extract was again centrifuged (12,000 x g for 5 min), and then frozen at -20° C until further use. The protein content of the extract was estimated following the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard. A protein concentration of 50µg ml⁻¹ was used for all assays.

4.2.2 Rearing of *B. amphitrite* larvae

The life cycle of *B. amphitrite* includes planktotrophic larval development consisting of six naupliar instars and a non-feeding cyprid instar. The first instar nauplii do not feed and molt into the second instar within 1-2 hours. Instars II to VI are phytoplanktotrophic. Nauplii were mass reared in 2-liter glass beakers using filtered seawater of 35‰, on a diet of *C. calcitrans*, a unicellular diatom, at a cell concentration of 2 x 10⁵ cells ml⁻¹. The feed

organism was replenished every day while changing water. After 5-6 days the cyprids obtained were siphoned out and stored at 5° C prior to settlement assays. Two-day-old cyprids were used to carry out the assays. These methods have been described in detail (Rittschof et al. 1984).

4.2.3 Isolation of bacteria from the shell surfaces of *B. amphitrite*

B. amphitrite brought to the laboratory were scraped with a nylon brush using Millipore filtered seawater under sterile conditions. The sample was further diluted and spread plated on Zobell Marine Agar 2216. The bacterial colonies thus isolated were maintained on Zobell Marine Agar 2216 slants. The purity of the culture was checked by streaking on Zobell Marine Agar 2216. The isolated bacteria were identified following Bergey's manual of systematic bacteriology (Krieg 1984).

4.2.4 Bacterial film

The influence of single-species films of *P. aeruginosa*, *B. pumilus* and *C. freundii* films was assessed at 35‰. Filming of the surfaces with bacteria was performed following the methods described by Maki et al. 1988,1990. The dishes with the bacterial treatment were fixed with formaldehyde (final concentration 1 to 2%; v/v) and the density of attached bacteria was determined using acridine orange and epifluorescence microscopy (Daley and Hobbie 1975). The adsorption technique resulted in densities of 10^6 to 10^7 attached bacteria per cm^2 .

4.2.5 Extraction of planktonic EPS

All three bacteria grown in marine broth (MB) were harvested by centrifugation (20,000 x g for 30 min) and subsequently sterile filtered (0.22 μ m, Millipore). The culture supernatant thus obtained was treated with 5 volumes of absolute ethanol and left at 4° C overnight. The precipitate (EPS) was recovered by centrifugation at (30,000 x g for 15 min) at 4° C, redissolved in distilled water and treated with DNase and RNase A (1-2 μ g ml⁻¹ final concentration) for three hours at 37° C. The material was dialyzed (8,000 MW cut-off) overnight at 4° C against distilled water and centrifuged at (30,000 x g for 20 min) at 25° C to remove insoluble material. The supernatant was recovered and lyophilized.

4.2.6 EPS characterization

The EPS was characterized for monosaccharide composition. For this, the EPS was hydrolyzed with 2N HCl for 2h at 100° C in ampoules flushed with nitrogen before sealing. After hydrolysis, the solution was evaporated to dryness under reduced pressure at 40° C. The neutral sugars were converted to alditol acetates and were analyzed by capillary gas chromatography (GC) as described earlier (Bhosle et al. 1995; Khandeparker and Bhosle 2001) (Chrompack, Middleburg, The Netherlands, Model CP 9002) equipped with a fused silica capillary column coated with CP sil-88 (25m, id=0.32mm, df=0.12; Chrompack, Middleburg, The Netherlands). A flame ionization detector was used to separate the alditol acetate mixture. A known amount (0.4 μ l) of the sample was applied using an on-column injector when the oven temperature

was 70° C. The oven temperature was rapidly raised to 150° C and further programmed at 3° C min⁻¹ to 230° C and maintained at this temperature for ca 10 min. Quantification of the components was done by peak area integration of the GC results using the data handling system installed in the instrument. The response factors were calculated using standard sugar alditol acetates and myoinositol as an internal standard and were used for the quantification of the results.

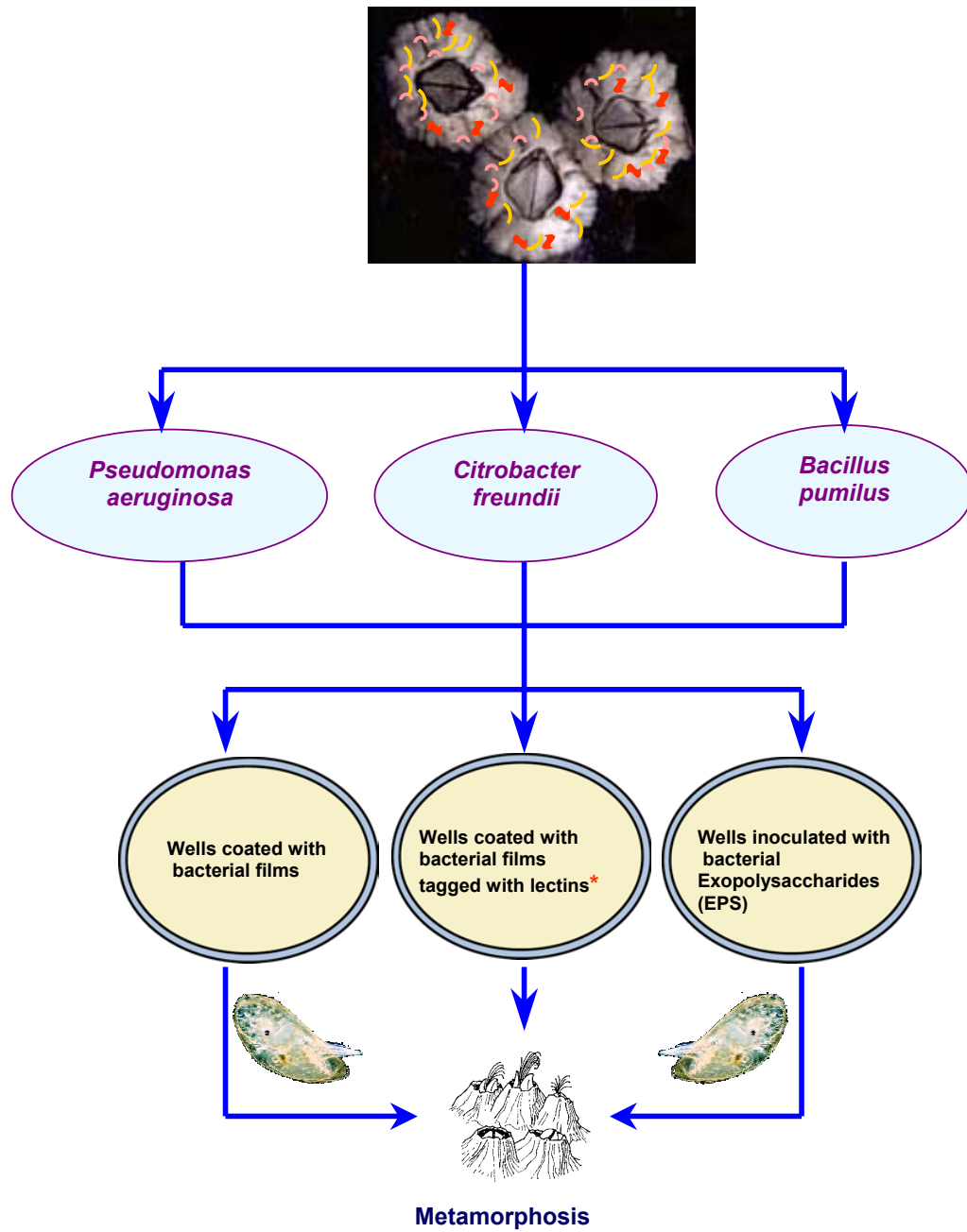
4.2.7 Treatment of bacterial films with lectins

The lectins, *Lens culinaris* agglutinin (LCA), Concanavalin A (ConA), *Limulus polyphemus* (Limulin), Wheat germ agglutinin (WGA) and Glycine max were obtained from Sigma chemicals each conjugated with FITC (fluorescein isothiocyanate).

The bacterial films of *P. aeruginosa*, *B. pumilus* and *C. freundii* were treated with 250µl of the lectin (stock solution of 1 or 5 mg/ml in distilled deionised water) as described by Hood and Schmidt (1996). After incubation for 30 min the multiwells were rinsed with autoclaved filtered seawater thrice to which ~25-30 cyprids were introduced with 5 ml of autoclaved filtered seawater of 35‰.

4.2.8 Assay protocol

The schematic representation of the experimental set-up is given in Figure 4.1.



**Lens culinaris* agglutinin (LCA); Concanavalin A (Con A); *Limulus polyphemus* (Limulin); Wheat germ agglutinin (WGA); Glycine max (Gly max)

Fig. 4.1 The schematic representation of the experimental set-up

The multi-wells were inoculated with the bacteria and planktonic EPS ($50\mu\text{g ml}^{-1}$). The multi wells inoculated with the bacterial films were rinsed off after three hours by repeated rinsing with autoclaved filtered seawater under a laminar chamber. Subsequently ~25-30 cyprids were introduced with 5 ml of autoclaved filtered seawater of 35‰. All the experiments were repeated four times using four different batches of larvae with six replicates at each trial ($n=6$, with batch as an additional factor). Controls were filtered seawater (FSW, negative control) and adult extract (AE, positive control). The settlement assays were carried out using Corning-430343, 6 well multiwells and were monitored for a period of four days with an intermittent observation everyday. Assay wells were maintained at $26\pm 1^\circ\text{C}$ (12h light: 12h dark photoperiod). The films probed with lectins were examined using Olympus (CX40) microscope equipped with a V-LH50HG fluorescence attachment.

4.2.9 Statistical analysis

Data in the form of percentage of larval settlement was arcsine transformed to ensure normality of means and homogeneity of variances before subjecting to statistical analysis. The influence of lectin-treated bacterial films on the cyprid metamorphosis was evaluated using one-way analysis of variance (ANOVA) (Sokal and Rohlf 1981). A post ANOVA analysis was performed using Scheffe's test ($\alpha=0.05$). Those that did not meet the normality assumption were analyzed using non-parametric statistical analysis such as Mann-Whitney U-Test ($\alpha=0.025$). The analysis was performed by transforming the values to ranks and was used to compare the means between the two treatments.

4.3 Results

Among the three bacterial species isolated from shell surfaces of *B. amphitrite*, *P. aeruginosa* positively influenced metamorphosis of cyprids. The cyprids exposed to *P. aeruginosa* facilitated (44%) metamorphosis similar as that of AE (44%) at the end of day 1 (Fig. 4.2).

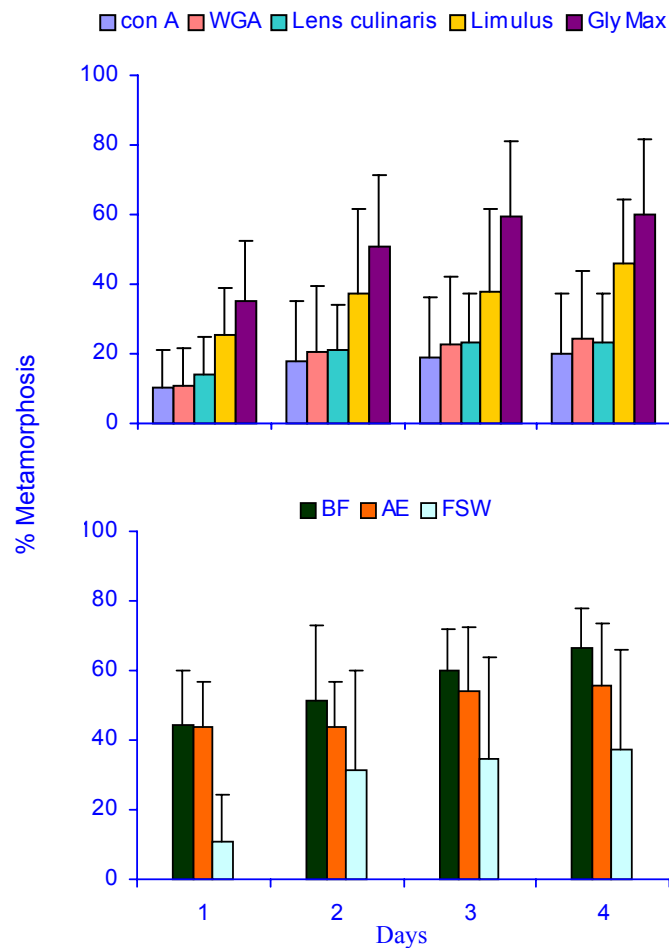


Fig. 4.2 Percentage metamorphosis of cyprids exposed to lectin-treated and non-treated films of *P. aeruginosa*. Vertical lines indicates the standard deviation from mean. AE- adult extract, FSW- filtered seawater, BF- bacterial film

C. freundii and *B. pumilus* films did not facilitate metamorphosis of cyprids (Figs. 4.3 and 4.4), the response was almost similar as that observed with plain filtered seawater (-ve control).

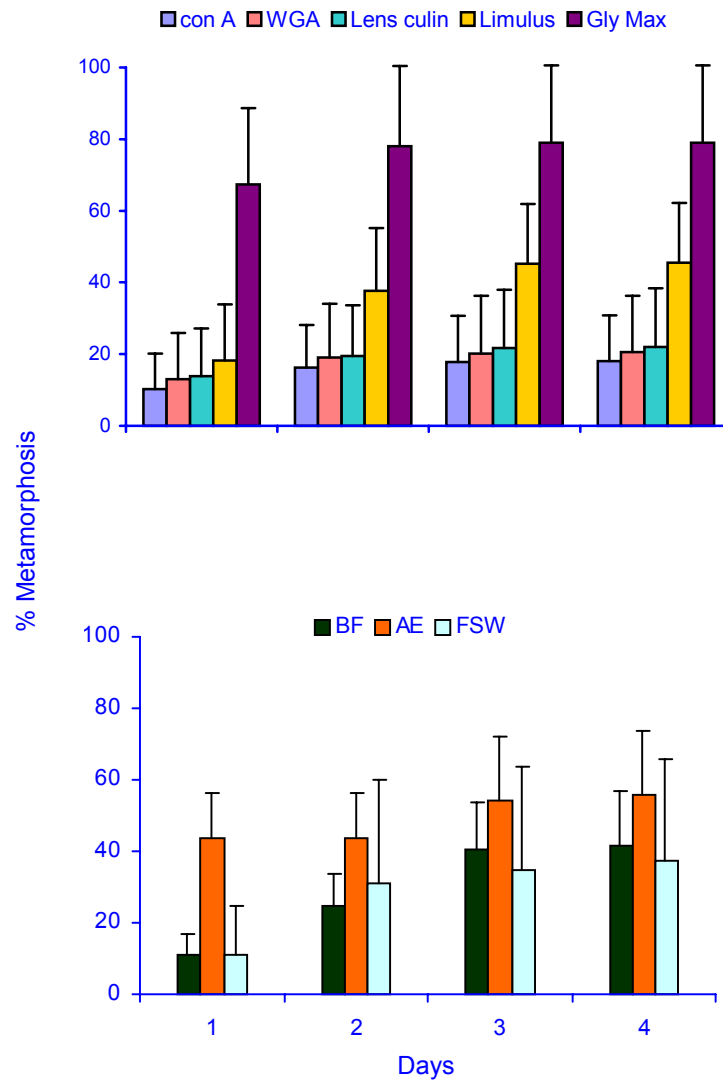


Fig. 4.3 Percentage metamorphosis of cyprids exposed to lectin-treated and non-treated films of *C. freundii*. Vertical lines indicates the standard deviation from mean. AE- adult extract, FSW- filtered seawater, BF- bacterial film

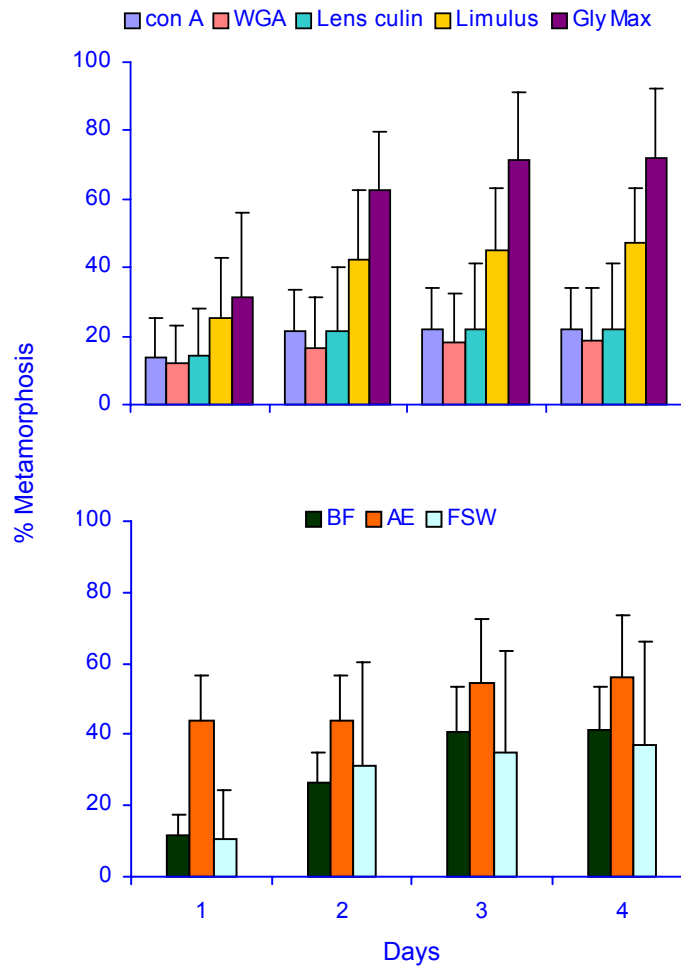


Fig. 4.4 Percentage metamorphosis of cyprids exposed to lectin-treated and non-treated films of *B. pumilus*. Vertical lines indicate the standard deviation from mean. AE- adult extract, FSW- filtered seawater, BF- bacterial film

The influence of planktonic or free exopolysaccharide (EPS) obtained from *P. aeruginosa* was comparatively more than the EPS produced by the other two bacteria on the metamorphosis of cyprids. However, the metamorphosis rates were lower than the *P. aeruginosa* films (Figs. 4.2 and 4.5). The EPS produced by *B. pumilus* and *C. freundii* were strongly inhibitory (Fig. 4.5).

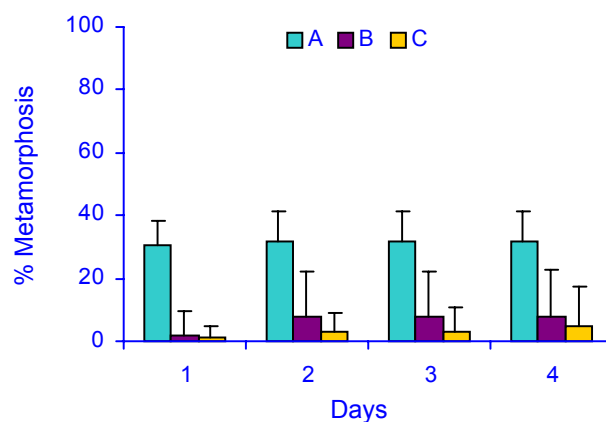


Fig. 4.5 Percentage metamorphosis of cyprids exposed to planktonic exopolysaccharides obtained from *P. aeruginosa* (A), *B. pumilus* (B) and *C. freundii* (C). Vertical lines indicate the standard deviation from mean

Capillary gas chromatography analysis result for the relative monosaccharide composition (%) of the planktonic EPS produced by all the three bacteria is presented in Table 4.1.

Table 4.1 Relative monosachharide composition (%) of the EPS produced by *P. aeruginosa*, *B. pumilus* and *C. freundii*.

| Microorganism | Mannose (%) | Glucose (%) | Ribose (%) |
|-------------------------------|-------------|-------------|------------|
| <i>Pseudomonas aeruginosa</i> | 86.28 | 13.71 | - |
| <i>Citrobacter freundii</i> | 80.17 | 14.95 | 4.86 |
| <i>Bacillus pumilus</i> | 86.17 | 13.10 | 0.72 |

All the three bacteria showed the presence of mannose and glucose, the former being the major constituent. *B. pumilus* and *C. freundii* showed the presence of ribose, which was absent in *P. aeruginosa*. The percentage of ribose in case of *B. pumilus* was less (0.72%) than that observed in *C. freundii* (4.86%).

The EPS intimately associated with the bacterial cell surface were probed through application of different lectins. The sugar specificity of different lectins used in the present experiment is shown in Table 4.2. The cyprids exposed to *P. aeruginosa* films treated with lectins, Con A, WGA, LCA and Limulin showed a decline in metamorphosis. The treatment of Glycine max to the films of *P. aeruginosa* showed almost similar metamorphosis response as that observed with untreated bacterial films ($p \leq 0.025$, Mann-Whitney).

Table 4.2 Sugar specificity of lectins

| Abbreviation lectin | Common name | Sugar specificity |
|---------------------------|----------------------------------|--|
| Con A | Concanavalin A | D-mannose D-glucose Fructofuranose |
| LCA | <i>Lens culinaris</i> agglutinin | D-mannose D-glucose |
| WGA | Wheat germ agglutinin | N-acetyl-D-glucosamine |
| <i>Limulus polyphemus</i> | Limulin | N-acetyl-D-glucosamine N-acetylneuraminic acid Glucuronic acid |
| Gly max | Glycine max | Phosphorycholine analogs N-acetyl-D-galactosamine |

A significant difference in the metamorphosis rates was observed (Table 4.3) when the bacterial films were treated with different lectins ($p \leq 0.001$, one-way ANOVA; $p \leq 0.05$, Scheffe's test).

The films of *C. freundii* did not facilitate metamorphosis of cyprids, but resulted in metamorphosis promotion higher than that observed with AE when treated with Glycine max (67%) at the end of day 1. The difference in the

metamorphosis rates between the untreated bacterial film and Glycine max treated film was highly significant ($p \leq 0.025$, Mann-Whitney).

Table 4.3 One-way ANOVA. Influence of lectin-treated and non-treated bacterial films (Treatment) of *P. aeruginosa*, *B. pumilus* and *C. freundii* on the metamorphosis of *B. amphitrite* cyprids. (df.degree of freedom; SS. sum of the squares; MS. mean of squares; Fs.Fischer constant).

| | <i>Pseudomonas aeruginosa</i> | | | | <i>Bacillus pumilus</i> | | | <i>Citrobacter freundii</i> | | |
|--------------------|-------------------------------|-------|------|---------|-------------------------|------|---------|-----------------------------|------|---------|
| | df | SS | MS | Fs | SS | MS | Fs | SS | MS | Fs |
| Day 1 | | | | | | | | | | |
| Treatment | 5 | 4788 | 958 | 9***** | 3580 | 716 | 9***** | 15486 | 3097 | 26***** |
| Within sub.Gr.err. | 138 | 14564 | 105 | | 10707 | 78 | | 16332 | 118 | |
| Total | 143 | 19352 | | | 14287 | | | 31818 | | |
| Day 4 | | | | | | | | | | |
| Treatment | 5 | 18662 | 3732 | 26***** | 23377 | 4675 | 36***** | 36634 | 7327 | 47***** |
| Within sub.Gr.err. | 138 | 19927 | 144 | | 17705 | 128 | | 21557 | 156 | |
| Total | 143 | 38589 | | | 41082 | | | 58191 | | |

(***** $p \leq 0.001$)

The films of *B. pumilus* treated with Glycine max and Limulin positively influenced metamorphosis while untreated films did not. The inducement by Glycine max-treated *B. pumilus* films was not comparable with *C. freundii* (32 and 67%) and was less than the AE (44%). However, the films treated with Glycine max showed a considerable increase in metamorphosis on day 2 (63%).

A marginal increase in the metamorphosis rates was observed as the cyprids as well as the bacterial films aged. However the pattern almost remained same except in case of *B. pumilus* where a considerable hike in metamorphosis was seen in the Glycine max-treated films.

4.4 Discussion

Epifluorescence microscopy coupled with the lectin specificity has been suggested as a new way to probe rapidly and precisely spatial relationships of complex distributions of molecules on marine surface when films are present (Michael and Smith 1995). Among the bacteria isolated from the shell surface of *B. amphitrite*, *P. aeruginosa* facilitated metamorphosis. However, when probed with Concanavalin A, WGA and LCA, which binds specifically to glucose and its derivatives, mannose and fructofuranose, negated the promotory effect. Hence, these carbohydrate moieties associated with the cell surface of *P. aeruginosa* are suggested to be involved in the larval induction of *B. amphitrite*. A decrease in the metamorphosis was also evident in case of *P. aeruginosa* films treated with Limulin suggesting the presence of N-acetylneuraminic acid, glucuronic acid or phosphocholine analogues in the EPS that could be involved in attracting the cyprids. However when probed with Glycine max there was not much difference in the metamorphosis response when compared to an untreated bacterial film indicating either absence or non-involvement of galactose derivatives associated with its EPS.

The films of *B. pumilus* and *C. freundii* did not promote metamorphosis. Whereas the Glycine max-treated bacterial films of *B. pumilus* and *C. freundii* showed a considerable increase in the metamorphosis, the metamorphosis rates being higher than that observed with the AE. Hence the probability of N-acetyl-galactosamine being associated with the capsular EPS of these bacteria cannot be ruled out. The blocking of the galactose derivatives on the films of *B. pumilus* and *C. freundii* enhanced the settlement, which were otherwise inhibitory to the cyprids of *B. amphitrite*. Three galactose-binding

lectins have been described in the haemolymph of the acorn barnacle *M. rosa* (Marques and Baracco 2000). The lectin present in the haemolymph of *B. rostrata* has been also reported to recognize the same monosaccharide (Toda et al. 1998). The fact that primary urine production is the result of ultrafiltration of the haemolymph (White and Walker 1981) it is possible that the lectins could find their way to the surface via the excretory canal. Hence, the presence of lectins in these animals other than enhancing the phagocytosis of microorganisms could also possibly play an important role in altering the signals or cues and may be ecologically important. The immobilization of marine bacteria in hydrogels has been developed as a new concept for the prevention of macrofouling (Gatenholm et al. 1995). The inhibitory components of the bacteria for e.g. in this study galactose residues can thus be protected by immobilizing them in the hydrogels and could be of great help in antifouling technology which needs further validation.

When the planktonic EPS of *P. aeruginosa* was assessed against the cyprids, although metamorphosis was facilitated, it was not as effective as the EPS associated with the bacterial cell surface. An earlier investigation has reported that the response of the cyprids of *B. amphitrite* to bacteria and its products is regulated by both contact chemoreception and/or olfaction, depending on the properties of the settlement inducing compounds (Khandeparker et al. 2002). The planktonic EPS of *B. pumilus* and *C. freundii* were strongly inhibitory. Capillary gas chromatographic analysis of the planktonic EPS revealed the presence of glucose and mannose which occurred as the most abundant carbohydrates in all the three bacteria, whereas ribose was additionally present in *B. pumilus* and *C. freundii*. The presence of ribose in the EPS may

be a responsible component for strong inhibitory effect of EPS produced by *B. pumilus* and *C. freundii*.

Aged cyprids showed higher rates of metamorphosis. A possible explanation could be that young cyprids are more discriminating than old cyprids but become less discriminating with age during settlement (Rittschof et al. 1984; Crisp 1988) presumably due to decline in their energy reserves and thus the physiological quality (Anil et al. 2001).

As all three bacteria were isolated from the shell surface of *B. amphitrite*, their involvement in the gregarious behavior cannot be ruled out. The presence of both facilitatory and inhibitory bacteria may have some adaptive implications wherein the adult barnacle avoids the epibiosis on some of its parts e.g. tergum and scutum / apical region from where the barnacle cirri open out for feeding, respiration etc.

In the present investigation the bacteria were assessed separately, whereas, in nature they occur in conjunction and the contradictory signals from each bacterium would give different set of signals to the settling larvae. Spatial and chemical heterogeneities of surfaces in marine environment have been illustrated by lectin probes and its role in possible microscale cues for biofouling is suggested (Michael and Smith 1995). The understanding of the synchronization of all the contradictory signaling molecules in presence of each other needs further validation. It has been reported earlier that in *B. amphitrite* cyprids the degree of inducement of metamorphosis varied with various combinations of exopolymers of different bacterial strains with or without AE (Anil et al. 1997; Anil and Khandeparker 1998). Some bacteria in

biofilms are capable of genetic exchange (Fry and Day 1990). The cell-cell communication between their own species and perhaps others through N-acyl-L-homoserine lactones (AHL) cannot be ruled out as AHL activity has been demonstrated in natural biofilms (McLean et al. 1997). The ability of larval receptors to distinguish between the EPS that are intimately associated with the cell surface and those are released in the planktonic form would be a key for production of probes for such saccharides and the genes that produce them can then be explored.

Chapter 5

Influence of larval rearing conditions and ageing on the energetics and metamorphosis

5.1 Introduction

It has been suggested that larval experiences other than predation may have an impact on recruitment. Though metamorphosis was successful, competitive ability was impaired due to reduced growth rate (Jarrett and Pechenik 1997). Pechenik et al. (1993) observed that to delay metamorphosis may be a double-edged sword for *B. amphitrite*, increasing an individual's chance of locating a site appropriate for metamorphosis, but simultaneously reducing the ability to compete for space during the first few weeks of juvenile life. The cypris larvae are non-feeding and have to depend upon the energy reserves incorporated during the earlier planktotrophic naupliar development.

Nucleic acids play a major role in growth and development. It has been shown that the RNA:DNA ratio is an indicator of nutritional condition (Clemmesen 1996) and has been routinely used for estimating growth rates or nutritional condition of larval fish in the field. Only a few studies have applied this technique to crustaceans and their larvae (Dagg and Littlepage 1972; Sulkin 1975; Anger and Hirche 1990; Wagner et al. 1998). In this study, the influence of rearing temperature and food concentration on ensuing changes in the content of RNA and DNA content of cyprids is examined. Ageing cyprids at 5 °C was adopted as it has been shown that the major cyprid protein content remained constant at this temperature (Satuito et al. 1996). The impact of these factors on the metamorphosis of the cypris larvae is presented.

5.2 Materials and methods

5.2.1 Rearing of *B. amphitrite* larvae

Larval development consists of six naupliar instars and a non-feeding cypris instar. The first nauplius stage is non-feeding and is of short duration. Nauplii obtained from *B. amphitrite* were mass reared in 2 L. glass beakers (1 larva ml⁻¹) on a daily diet of *C. calcitrans*, a unicellular diatom, at a concentration of 1 x10⁵ and 2 x10⁵ cells ml⁻¹. The cultures were maintained at 20 and 30° C (±1° C) in an incubator (12h light:12h dark photoperiod). The larval density and food concentrations were maintained by monitoring mortality of the larvae every day. The rearing experiments for each of the conditions were carried out three times.

5.2.2 Cyprid metamorphosis assay

The cyprids obtained were filtered through a tier of plankton netting (300, 230 and 160µm). Cyprids (~10) were sampled for their length and breadth measurements as and when they appeared. The cyprids obtained on day 5 at 30 °C (2 x10⁵ cells ml⁻¹), day 6 at 30° C (1 x10⁵ cells ml⁻¹) and day 10 at 20° C (2 x10⁵ and 1 x10⁵ cells ml⁻¹) were used in metamorphosis assays.

The adult extract (AE) which is known to promote metamorphosis in *B. amphitrite* cyprids was used as a positive control and was prepared following the method described by Larman et al. (1982). In addition, cyprid metamorphosis was also monitored in the absence of the adult extract (control). The protein content of the extract was estimated using the method described by Lowry et al. (1951) using BSA (Bovine Serum Albumin) as the standard. A concentration of 50 µg ml⁻¹ was used in the assays. The assays in

each condition were run in triplicates. About 20-25 cyprids were introduced into polystyrene multiwells (Nunclon delta 1-52795) with 5ml of filtered, autoclaved seawater (35‰). Assays were carried out at $25\pm 1^{\circ}$ C.

5.2.3 Estimation of nucleic acids in *B. amphitrite* larvae

The technique described by Clemmesen (1988,1993), which is based on fluorimetric determination of RNA and DNA content of an individual fish larva, was adopted for estimation of nucleic acids in the larvae of *B. amphitrite*. All the chemicals used were analytical grade. Ethidium bromide was obtained from BDH laboratories and Ribonuclease A was obtained from Merck. Yeast ribonucleic acid (RNA) and Calf thymus deoxyribonucleic acid (DNA) were procured from Hi media. Sodium dodecyl sulphate was procured from Sigma chemicals (USA). Standard regression curves of DNA and RNA were plotted. Larval homogenate (10 in no) was prepared, and then spiked with known amounts of standard DNA and RNA to evaluate the precision of the method, which was found to be $\pm 5\%$.

5.2.4 Statistical analysis

Influence of rearing temperature and food concentration on the size (total length and total breadth) of cyprids, DNA and RNA content and RNA:DNA ratio were evaluated by two-way ANOVA (Sokal and Rohlf 1981).

5.3 Results

The cyprids formed at 30° C had total length ranging from 451 to 480 µm and total breadth from 218 to 227 µm and were larger than those formed at 20° C, which had total length ranging from 421 to 448 µm and total breadth from 211 to 230 µm.

Two-way ANOVA showed that the food concentration and temperature influenced cypris length and breadth significantly. The food concentration and temperature interaction showed an insignificant effect indicating the influence to be equal at both the temperature and food concentration (Table 5.1).

Table 5.1 Two-way ANOVA of the influence of food concentration and temperature on cyprid length, cyprid breadth, DNA, RNA and RNA/DNA of cyprids.

| Factor | df | Cyprid length | | | Cyprid breadth | | |
|---------------------|----|---------------|--------|-----------|----------------|------|---------|
| | | SS | MS | Fs | SS | MS | Fs |
| Food Conc. | 1 | 5198 | 5198.4 | 26.5***** | 1172 | 1172 | 5.19* |
| Temperature | 1 | 15920 | 15920 | 81.3***** | 2030 | 2030 | 9.0**** |
| Food conc.x Temp | 1 | 636 | 636.8 | 3.25 ns | 159 | 159 | 0.70 ns |
| Within sub. Gr.err. | 36 | 7043 | 195.6 | | 8122 | 225 | |
| Total | 39 | 28797 | | | 11483 | | |

| Factor | df | DNA | | | RNA | | | RNA/DNA | | |
|---------------------|----|-------|-------|---------|------|------|---------|---------|------|--------|
| | | SS | MS | Fs | SS | MS | Fs | SS | MS | Fs |
| Food Conc. | 1 | 0.03 | 0.03 | 0.3 ns | 0.79 | 0.79 | 3.09 ns | 0.01 | 0.01 | 0.07ns |
| Temperature | 1 | 0.34 | 0.34 | 3.05 ns | 0.96 | 0.96 | 3.78 ns | 0.14 | 0.14 | 0.75ns |
| Food conc.x Temp | 1 | 0.007 | 0.007 | 0.06 ns | 2.17 | 2.17 | 8.5*** | 0.36 | 0.36 | 1.99ns |
| Within sub. Gr.err. | 28 | 3.16 | 0.11 | | 7.17 | 0.25 | | 5.19 | 0.18 | |
| Total | 31 | 3.55 | | | 11.1 | | | 5.71 | | |

(*****p≤0.001, ****p≤0.005, ***p≤0.01, *p≤0.05, ns- not significant)

The average DNA content of the cyprids from the different rearing conditions (Figs. 5.1a,e and 5.2a,e; 0 day values) averaged 1.9 to 2.1 µg larva⁻¹. The RNA content averaged 3.2 µg larva⁻¹ (2x10⁵ cells ml⁻¹) and 2.4 µg larva⁻¹ (1x10⁵ cells ml⁻¹) at 30° C (Figs. 5.1b and 5.2b), whereas at 20° C the average RNA content was 2.3 µg larva⁻¹ at 2x10⁵ cells ml⁻¹ and 1.7 µg larva⁻¹ at 1x10⁵ cells ml⁻¹ (Figs. 5.1f and 5.2f).

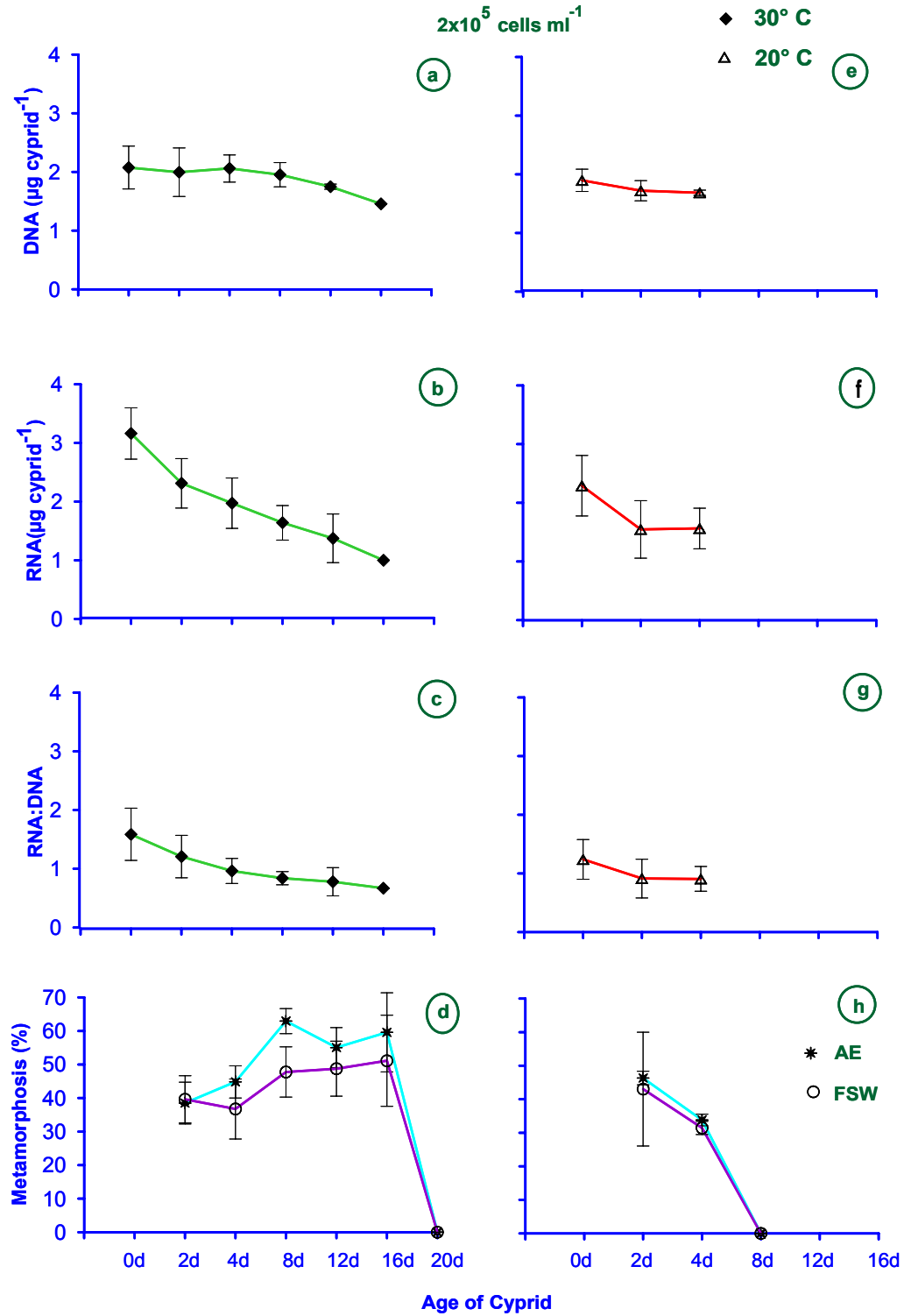


Fig. 5.1 Influence of ageing of cyprids at 5° C on DNA, RNA and RNA/DNA ratio and percentage metamorphosis. (a), (b), (c) and (d) represent larvae raised at 30° C and (e), (f), (g) and (h) represent larvae raised at 20° C at $2 \times 10^5 \text{ cells ml}^{-1}$

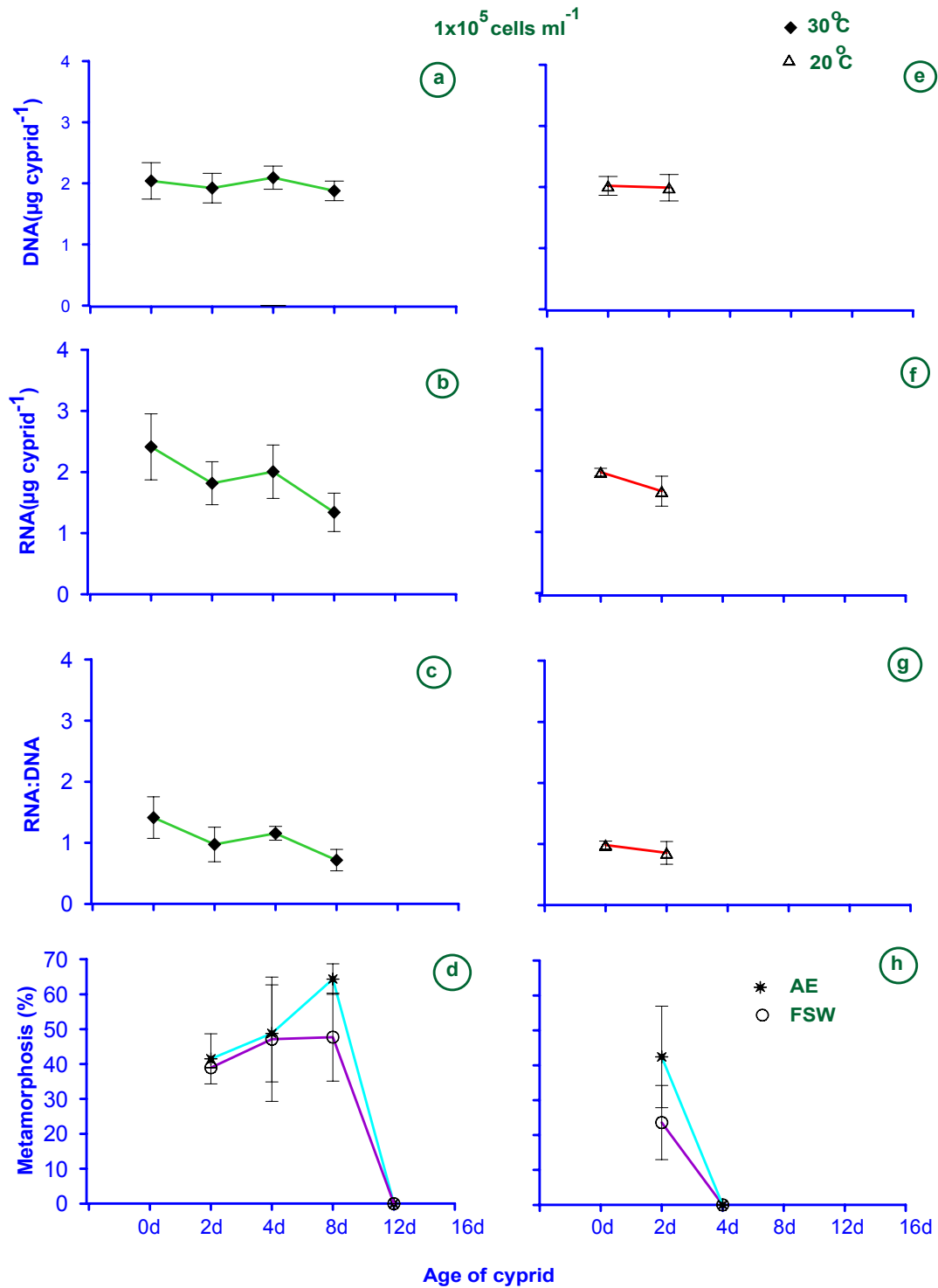


Fig. 5.2 Influence of ageing of cyprids at 5°C on DNA, RNA and RNA/DNA ratio and percentage metamorphosis. (a), (b), (c) and (d) represent larvae raised at 30°C and (e), (f), (g) and (h) represent larvae raised at 20°C at 1×10^5 cells ml⁻¹

The cyprids on aging at 5° C showed that RNA (Figs. 5.1b,f and 5.2b,f) and the RNA:DNA ratio progressively decreased (Figs. 5.1c,g and 5.2c,g). The cyprid attachment assay (Figs. 5.1d and 5.2d) results, evaluating the influence of aging showed that the larvae reared at 30° C, 2×10^5 cells ml⁻¹ and 1×10^5 cells ml⁻¹, were viable for 16 and 8 days respectively. The metamorphosis rate peaked with 8-16 day aged cyprids and was positively influenced by AE when compared to the control (FSW) (Figs. 5.1d and 5.2d).

The cyprids raised at 20° C could only metamorphose successfully for 4 days (2×10^5 cells ml⁻¹) and 2 days (1×10^5 cells ml⁻¹). The metamorphosis rate decreased with aging and was similar in both AE and control conditions (Figs. 5.1h and 5.2h).

5.4 Discussion

Spawning activity in some marine invertebrates coincides with phytoplankton blooms (Barnes and Barnes 1958; Starr et al. 1990). This coupling is explained as one of the survival measures offered to the planktotrophic offspring. Barnes and Barnes (1958), while examining the recruitment of *Balanus* (= *Semibalanus*) *balanoides* over a period of ten years in the Firth of Clyde, attributed recruitment failure to abnormal increase in larval predation, unusual physical parameters, presence of deleterious chemicals as well as defective sampling. Olson and Olson (1989), however, felt that recruitment largely depended on the abundance of larval food. Cyprids like other invertebrate larvae derive their energy from stored lipids. The extent of this energy reserve in cyprids depends on the condition under which the nauplii grow (Lucas et al. 1979). In the case of cyprids, both food concentration and

temperature influenced length and breadth. This fact coupled with the information that larvae take longer duration to complete development at the lower temperature indicate that assimilation of energy for growth is significantly influenced by these two variables.

Cypris, the terminal larval instar, prolong their larval duration until a conducive substratum is available for settlement and subsequent metamorphosis. Freeman and Costlow (1983) showed that moulting is distinctly biphasic in cyprids of *B. amphitrite*. In free-swimming cyprids moulting is arrested at an early stage and is only resumed after settlement. Pechenik et al. (1998) state that growth rate of newly-metamorphosed barnacles slows down significantly if *B. amphitrite* cyprids were prevented from metamorphosing for as few as 3 days. It was observed by Connell (1961) that reduced growth rates would compromise the ability of juvenile barnacles to compete for space and seriously reduce their likelihood of successfully recruiting to the adult population. In the light of these observations, Jarrett and Pechenik (1997) and Pechenik et al. (1998) opined that larval experience influences juvenile performance. The evidence gathered from the present experiments indicate that the naupliar experience clearly determines the capability of the cyprids to metamorphose as well. The RNA content of larvae raised at 20° C, which had longer total naupliar duration, was considerably less compared to those raised at 30° C. This difference in RNA content was reflected in the capability of the cyprids to survive ageing at 5° C. The larvae raised at 20° C could only successfully metamorphose until 2 to 4 days, whereas, those raised at 30° C could do so for 8 to 16 days. Clare et al. (1994) observed that the cyprid settlement of *B. amphitrite* is uncharacteristically low when reared with *D.*

tertiolecta and that, although the cyprids look normal, they may be deficient in some respect. It was also observed earlier that, by day 3, the cyprids lose their power to discriminate the substrata and are less useful for assaying metamorphosing inducers (Rittschof et al. 1984; Crisp 1988; Clare et al. 1994). Maki et al. (1988) showed from their experimental results on inhibition of attachment of *B. amphitrite* to bacterial films, 4 day old cyprids showed an increase in larval attachment as compared to 2 day old cyprids. It was observed with cyprids of *Balanus* (= *Semibalanus*) *balanoides* after 4-5 weeks at 10° C a final threshold level was reached wherein they possessed insufficient energy to accomplish metamorphosis into a feeding juvenile barnacle (Lucas et al. 1979). Høeg and Ritchie (1987) observed with *Lernaeodiscus porcellanae* (Cirripedia: Rhizocephala) larvae, which have lecithotrophic nauplii and small sized cyprids, the energy threshold, where metamorphosis was no longer possible, was seemingly reached in less than 15 days and attributed this to the rearing temperature. The results of the present investigation show cypris metamorphosis rate and the influence of ageing are governed by naupliar rearing conditions and will be of critical importance to recruitment and early post-settlement mortality.