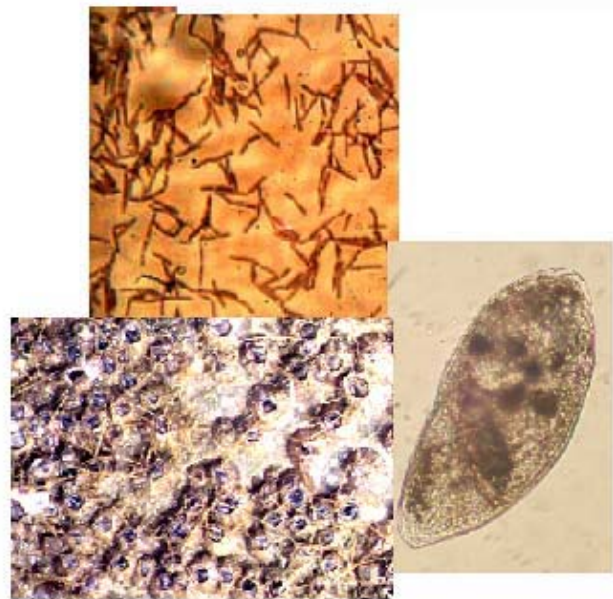


Studies on some cues regulating
metamorphosis of the larvae of
Balanus amphitrite (Cirripedia:Thoracica)



Lidita D.S. Khandeparker



National Institute of Oceanography
Council of Scientific & Industrial Research
Dona Paula, Goa - 403 004, INDIA



August 2002

Studies on some cues regulating
metamorphosis of the larvae of
Balanus amphitrite (Cirripedia:Thoracica)

Thesis Submitted to the
Goa University
for the degree of
Doctor of Philosophy in
Marine Science

Lidita D. S. Khandeparker



National Institute of Oceanography
Council of Scientific & Industrial Research
Dona Paula, Goa - 403 004, INDIA



August 2002

Contents

| | Page |
|--|------|
| Statement | |
| Certificate | |
| Acknowledgements | |
| Chapter 1. General Introduction | 1 |
| Chapter 2. Significance of sugars in exploration and metamorphosis | |
| 2.1 Introduction | 10 |
| 2.2 Materials and methods | 13 |
| 2.2.1 Preparation of adult extract (AE) | 13 |
| 2.2.2 Rearing of <i>B.amphitrite</i> larvae | 13 |
| 2.2.3 Treatment with sugars | 14 |
| 2.2.4 Visualization of footprints | 14 |
| 2.2.5 Assay protocol for evaluation of metamorphosis | 15 |
| 2.2.6 Statistical analysis | 16 |
| 2.3 Results | 17 |
| 2.4 Discussion | 21 |
| Chapter 3. Evaluation of different inducers from microorganisms that influence the metamorphosis | |
| 3.1 Introduction | 25 |
| 3.2 Materials and methods | 28 |
| 3.2.1 Preparation of the adult extract (AE) | 28 |
| 3.2.2 Rearing of <i>B. amphitrite</i> larvae | 29 |
| 3.2.3 Isolation of bacteria from shell surfaces of <i>B. amphitrite</i> | 29 |
| Experiment 1 Influence of surface-bound compounds of bacteria and its products on metamorphosis. | 33 |
| 3.2.4 Bacterial film (BF) | 33 |
| 3.2.5 Culture supernatant and its fractions | 33 |
| 3.2.6 Bacterial extract | 34 |
| Experiment 2 Effectiveness of leachants in presence of surface-bound compounds on metamorphosis. | 34 |
| Experiment 3 Influence of culture supernatants extracted using different nutrient mediums on metamorphosis. | 35 |
| 3.2.7 Semi-solid culture | 35 |

| | | |
|---|--|-----|
| | Experiment 4 Influence of bacterial exopolysaccharides extracted using different nutrient mediums on metamorphosis. | 36 |
| 3.2.8 | Extraction of bacterial exopolysaccharides (EPS) | 36 |
| 3.2.9 | FTIR spectroscopy | 37 |
| 3.2.10 | Assay protocol for Experiments 1,2,3 and 4 | 37 |
| 3.2.11 | Assay protocol for thraustochytrid (# MS 2D) | 38 |
| 3.2.12 | Statistical analysis | 38 |
| 3A | <i>Pseudomonas aeruginosa</i> | 39 |
| 3A 3 | Results | 39 |
| 3A 3.1 | Experiment 1 | 39 |
| 3A 3.2 | Experiment 2 | 48 |
| 3A 3.3 | Experiment 3 | 49 |
| 3A 3.4 | Experiment 4 | 52 |
| 3A 3.5 | FTIR spectroscopy | 53 |
| 3B | <i>Bacillus pumilus</i> | 55 |
| 3B 3.1 | Experiment 1 | 55 |
| 3B 3.2 | Experiment 2 | 64 |
| 3B 3.3 | Experiment 3 | 65 |
| 3B 3.4 | Experiment 4 | 68 |
| 3B 3.5 | FTIR spectroscopy | 68 |
| 3C | <i>Citrobacter freundii</i> | 72 |
| 3C 3.1 | Experiment 1 | 72 |
| 3C 3.2 | Experiment 2 | 80 |
| 3C 3.3 | Experiment 3 | 81 |
| 3C 3.4 | Experiment 4 | 84 |
| 3C 3.5 | FTIR spectroscopy | 85 |
| 3D | Thraustochytrid (#MS 2D) | 88 |
| 3.4 | Discussion | 89 |
| Chapter 4. Lectins as probes to evaluate the role of signaling molecules from the bacteria | | |
| 4.1 | Introduction | 97 |
| 4.2 | Materials and Methods | 100 |
| 4.2.1 | Preparation of adult extract (AE) | 100 |
| 4.2.2 | Rearing of <i>B. amphitrite</i> larvae | 100 |
| 4.2.3 | Isolation of bacteria from the shell surfaces of <i>B. amphitrite</i> | 101 |
| 4.2.4 | Bacterial film | 101 |
| 4.2.5 | Extraction of planktonic EPS | 102 |
| 4.2.6 | EPS characterization | 102 |
| 4.2.7 | Treatment of bacterial films with lectins | 103 |

| | | |
|--|---|------------|
| 4.2.8 | Assay protocol | 103 |
| 4.2.9 | Statistical analysis | 105 |
| 4.3 | Results | 106 |
| 4.4 | Discussion | 112 |
| Chapter 5. Influence of larval rearing conditions and aging on the energetics and metamorphosis | | |
| 5.1 | Introduction | 116 |
| 5.2 | Materials and Methods | 117 |
| 5.2.1 | Rearing of <i>B. amphitrite</i> larvae | 117 |
| 5.2.2 | Cyprid metamorphosis assay | 117 |
| 5.2.3 | Estimation of nucleic acids in <i>B.amphitrite</i> larvae | 118 |
| 5.2.4 | Statistical analysis | 118 |
| 5.3 | Results | 119 |
| 5.4 | Discussion | 122 |
| Chapter 6. | Summary | 125 |
| | Bibliography | 132 |

Statement

As required under the University ordinance 0.19.8 (vi), I state that the present thesis entitled “**Studies on some cues regulating metamorphosis of the larvae of *Balanus amphitrite* (Cirripedia: Thoracica)**” is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

LIDITA D S KHANDEPARKER

Certificate

This is to certify that the thesis entitled “**Studies on some cues regulating metamorphosis of the larvae of *Balanus amphitrite* (Cirripedia: Thoracica)**”, submitted by Ms. Lidita D S Khandeparker for the award of the degree of Doctor of Philosophy in Marine Science is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any Universities or Institutions.

Dr. S. Raghukumar
Research Guide
Scientist
National Institute of Oceanography
Dona Paula – 403 004, Goa

Acknowledgements

I express my deep sense of gratitude to my guide Dr. S. Raghukumar, Scientist, National Institute of Oceanography, Goa, for his inspiring guidance, encouragement and support throughout the study.

I am equally grateful to Dr. A. C. Anil, Scientist and Head, MCMRD, National Institute of Oceanography, Goa, for his valuable guidance, suggestions and affectionate encouragements throughout the study period. I am also indebted to him for sharing his splendid experience and knowledge with me.

I am also thankful to Dr. C. U. Rivonkar, Lecturer, Goa University, for providing guidance and support during the study.

I am grateful to Dr. E. Desa, Director, National Institute of Oceanography for providing me the necessary laboratory facilities during the research work.

I am thankful to Council of Scientific and Industrial Research, India for the award of Senior Research Fellowship. I also thank Office of Naval Research, USA for support during the initial phases of the work.

Special thanks to Dr. A. B. Wagh for his encouragement.

I gratefully acknowledge the advise and support provided by Dr. N. B. Bhosle. I am also grateful to Dr. S. S. Sawant for providing generous help during my study.

I very much appreciate and gratefully acknowledge the efforts of Mr. K. Venkat during my research work as well as for the excellent job he has done during thesis compilation.

I am also indebted to Prof. Dr. U.M.X. Sangodkar, Dr. C. L. Rodrigues and Dr. G. N. Nayak of Goa University. I also acknowledge the help given by Dr. Lata Raghukumar, Dr. N Ramaiah and Dr. P. S. Parmeswaran.

I extend my sincere thanks to Dr. Ute Hentschel, Univ. of Würzburg, Germany for her help in bacterial identification.

I am thankful to Dr. N. L. Thakur, Ms. Smita Mitbavkar, Mr. Jagadish Patil, Mr. Y. Vishwakiran, Ms. Rakhee Khandeparker, Mr. Fraddry D'Souza, Dr. Prasanna Yennawar, Mr. P V Bhaskar, Ms. Brenda Fernandes, Ms. Ranee Veigas, Ms. Seema Araligidad, Ms. Priya D'Costa for their help during various stages of my research work.

I express my sincere thanks to Mr. Shyam Naik, Mr. N. S. Prabhu, Mr. A. P. Selvam and Mr. P.R. Kurle for their ever-helping attitude.

I acknowledge the help rendered by Ms. Anita Garg, Mr. Shripad Kunkolienkar, Ms. Vrushali Kolhe, Ms. Leena Prabhudesai, Ms. Sahana Hegde, Mr. Chetan Gaonkar for their cooperation during this study.

I am thankful to the staff of drawing section and workshop for all the help provided during the studies.

I am deeply indebted to my husband Dr. Dattesh V Desai for his constant encouragement and support without which this thesis would never have reached completion. I am greatly indebted to my parents, in-laws and all my family members.

(Lidita D S Khandeparker)

Chapter 1
General Introduction

General Introduction



On immersion of a surface in the marine environment the fundamental processes that contribute to the development of fouling communities are initiated. Fouling results in modification of the immersed structures leading to deterioration of its performance and is a complex mixture of physical, chemical and biological phenomenon. In general, the first stage is the adsorption of a conditioning film of organic and inorganic compounds to the surface. Conditioned surfaces are then colonized by various microorganisms such as bacteria, diatoms etc. (Marshall et al. 1971; Costerton et al. 1978) followed by attachment of algal spores and invertebrate larvae (Wahl 1989). These biofilms play an important role in mediating settlement and metamorphosis of invertebrate larvae.

Barnacles are dominant fouling organisms found all over the world and are the major target organisms in the development of antifouling technology. Among barnacles, *Balanus amphitrite* is an important model organism for these studies because of its rapid larval development, the ease of raising synchronous mass cultures and the predictable settlement in static conditions. *B. amphitrite* is euryhaline (Anil et al. 1995), breeds throughout the year (Karande 1967; Anil 1986) and is a dominant fouling organism in the fouling community in Indian waters (Karande 1967; Anil 1986; Venugopalan and Wagh 1990; Fernando 1990; Rao 1989; Santhakumaran 1989). It has been reported from both east and west coasts of India (Karande 1967,1974; Fernando 1978; Anil 1986; Pillai 1958).

The larval development of this organism includes six naupliar instars and a non-feeding pre-settling cyprid instar, specialized for exploration of a suitable surface for settlement and metamorphosis into the adult. Cypris larva is discriminating in its choice of settlement site (Knight-Jones 1953; Crisp and Meadows 1962, 1963). Laboratory and field studies have demonstrated that barnacle cyprids prefer to metamorphose on or near conspecifics. This gregarious feature has been related to settlement pheromone, a glycoprotein present in the adults, referred as arthropodin (Knight-Jones and Crisp 1953; Knight-Jones 1953; Crisp and Meadows 1963). Native barnacle pheromones are thought to be a heterogeneous group of 3,000 to 5,000 Dalton peptides (Rittschof 1985). Recently, a settlement-inducing protein complex (SIPC) from the adult barnacle, *B. amphitrite* has been isolated which is composed of three major subunits with molecular weights of 76, 88 and 98 kDa (Matsumura et al. 1998b). The settlement-inducing activity of the adult extract (AE), which has SIPC, was suppressed by lentil lectin (LCA), suggesting that the carbohydrate moiety of the adult glycoprotein is important in this species (Matsumura et al. 1998a).

Two types of barnacle adhesion to a substratum have been observed, namely temporary and permanent adhesion (Maki et al. 1994). In the marine environment the cyprid employs the antennular disc, an adhesive organ, for temporary attachment to the substratum (Nott 1969; Nott and Foster 1969). Barnacle cement is used for permanent settlement and is an underwater adhesive insoluble protein complex. Temporary attachment by the antennules retains the larva on the substratum and enables exploration to

take place (Walker et al. 1987). However, if a substratum proves unsuitable, cyprid can detach and swim-off to locate other surfaces. The various factors influencing settlement site selection are outlined by Crisp (1974), among which the chemical factors are sensed by antennular disc apical sense organ and terminal setae of fourth antennular segment (Fig. 1).

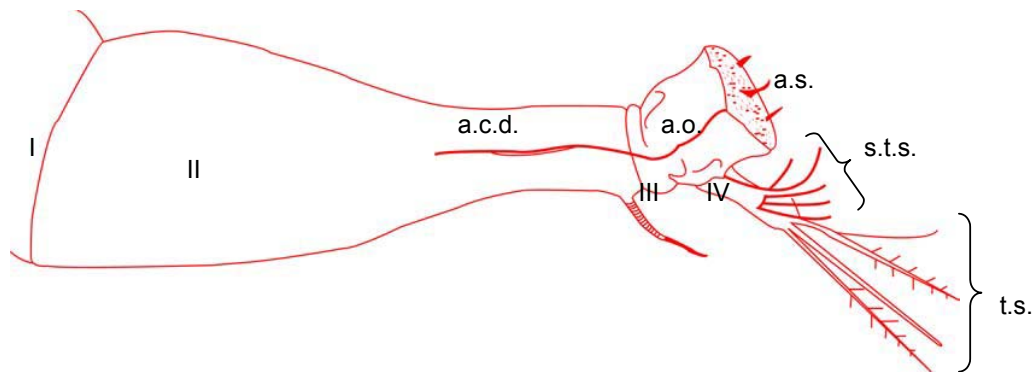


Fig. 1 Cypris antennule showing segments I-IV with attachment organ (a.o.) on third segment, a.c.d. – axial cement duct, a.s. – axial sensory seta, s.t.s – subterminal setae, t.s – terminal setae; Source: Walker G, Yule A.B. and Nott J.A. 1987, Barnacle biology: Crustacean Issues 5, A.A. Balkema, Rotterdam.

The barnacle cyprid is discriminating in its choice of settlement sites and in order to exercise its power of discrimination it has to explore the surface. The third antennular segment with its attachment disc is the most obvious point of contact between the cyprid and the substratum during the search (Nott 1969; Nott and Foster 1969). Darwin (1851,1854) observed that the attaching antennular segment consists of large, thin, circular sucking disc at the edge of which cement is secreted, and the antennular disc becomes attached to the substratum. The cyprid after settling and attachment to a surface molts its carapace and the body exoskeleton except for the embedded parts of the antennule after which it metamorphoses into an adult (Lindner 1984).

While exploring some surfaces, cyprids leave behind 'footprints' of temporary adhesive, which are believed to be secreted by the glands of the antennular disc. A relatively attractive substratum will acquire a correspondingly large number of footprints. The presence of footprints increase the attractiveness of a substratum and should result in gregarious settlement even in the absence of conspecific adults (Walker and Yule 1984; Yule and Walker 1985). However, the detection of settlement pheromone in the solution directs the attention to the fourth antennular segment that bears an impressive array of sensory setae (Gibson and Nott 1971; Clare and Nott 1994) as the putative site of pheromone reception. Flicking of the fourth antennular segment is evident while a cyprid explores a substratum (Clare et al. 1994). If a chemosensory mechanism of pheromone recognition is involved in the barnacle recruitment, there must be a signal transduction step in the pathway. Rittschof et al. (1986) suggested that settlement might be effected by an external protein/peptide receptor and a transduction pathway that involves the stimulation of adenylate cyclase. Yamamoto et al. (1995) demonstrated that a protein kinase C (PKC) signal transduction system plays an important role in larval metamorphosis of *B. amphitrite*. The evidence for the involvement of cyclic AMP (cAMP) in the settlement of this species is provided by Clare et al. (1995) and is in accord with mammalian olfaction for which cAMP is a signal transduction pathway component (Anholt 1991). Cyclic AMP acts as an intracellular signaling molecule in all prokaryotic and animal cells. It is synthesized from ATP by a plasma-membrane-bound enzyme adenylyl cyclase, and it is rapidly and continuously destroyed by one or more cyclic AMP phosphodiesterases, which hydrolyze cyclic AMP to adenosine 5'-

monophosphate (5'-AMP). Clare and Matsumura (2000) suggested that barnacle settlement induction involves receptor-ligand interactions and a signal transduction pathway(s) that translates into attachment and metamorphosis. However, larvae are likely to respond to more than one sensory stimulus when searching for a settlement location, and some factors such as naturally produced bacterial metabolites may override the importance of others (Maki et al. 1989).

In many cases the recognition of the chemical cues and other environmental stimuli by the larvae is suggested to be mediated by the larval nervous system (Bonar et al. 1990; Morse 1990). It has been illustrated that 5HT, DOPA and dopamine induce the settlement of cyprid larvae (Kon-ya and Endo 1995) thus suggesting that neurotransmitters (biogenic amines) are regulators of barnacle settlement and/or metamorphosis. The surface wettability has also been showed to rearrange the bacterial components that are exposed to the larvae.

Marine invertebrate larvae are presented with a wide range of cues as they approach a substratum. These cues may be physical ones or biologically derived chemical cues associated with bacteria, microflora and microfauna. Microbial biofilms have generally been examined as a stimulus for the settlement of macrofouling organisms (Crisp 1974). The larvae may use specific chemical signatures from biofilms or characteristic microbial assemblages to indicate preferred ecological conditions at a site. Films composed of individual strains of bacteria can have varying effects on larval

attachment (Kirchman et al. 1982a; Weiner et al. 1985,1989; Maki et al. 1988,1989; Szewzyk et al. 1991; Holmstrom et al. 1992; Avelin Mary et al. 1993; O' Connor and Richardson 1996). A bacterium may also elicit different responses by different fouling organisms. The bacterium, *Deleya marina*, stimulated the settlement of spirorbid polychaete larvae but inhibited the settlement of both bryozoan (Maki et al. 1989) and barnacle larvae (Maki et al. 1992). Maki et al. (1990) showed that the same bacterium when adsorbed on different substrata it elicited different attachment responses by barnacle larvae. Neal and Yule (1994) demonstrated that the age of the biofilm, rather than the surface wettability determined the larval adhesion. Barnacle cyprids like most other larvae prefer to settle on the substrata that possess a well-developed biofilm (Crisp 1984; Clare et al. 1992). The studies related to interactions between cypris larvae and bacterial films have generally found most bacterial species to inhibit attachment of *B. amphitrite* cyprids to polystyrene surfaces, although several bacterial species showed no effect (Maki et al. 1988,1990,1992; Avelin Mary et al. 1993; Neal and Yule 1994a,b).

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; Szewzyk et al. 1991; Maki et al. 1992). The bacteria can change the nature of the substratum either by altering the surface wettability or by exposing different surface molecular domains for example, in the form of exopolymers (Anil et al. 1997; Khandeparker et al. 2002). Exopolymers and other excreted products produced by microorganisms have been shown to be involved in settlement of macrofoulers, metamorphosis induction, growth and

development of organisms (Maki et al. 1990, 1992; Holmström et al. 1992,1996; Holmström and Kjelleberg 1994; Mary et al. 1993; Keough and Raimondi 1996). The structure of bacterial exopolymers is capable of either determining the effectiveness of the cypris temporary adhesive or affecting the cyprid's 'willingness to detach' (Yule and Walker 1984).

Natural microbial communities on estuarine and marine substrata, presumably containing a variety of bacterial species, can stimulate, inhibit, or have no effect on settlement (permanent attachment) of larval barnacles (Strathmann et al. 1981; Maki et al. 1988,1990). The period over which a natural microbial film has developed can also influence the settlement response of cyprids (Keough and Raimondi 1995; Weiczorek et al. 1995).

The involvement of lectins in the settlement and metamorphosis of invertebrates has been hypothesized for many years. Lectins, a class of naturally occurring proteins or glycoproteins exists in almost all living organisms and can recognize and bind carbohydrates specifically and noncovalently. Lectins play a key role in cell adhesion and processes, which involve specific recognition between cells during development (Frazier and Glaser 1979; Barondes 1980).

The lectin model was hypothesized after a series of experiments (Kirchman and Mitchell 1981; Kirchman et al. 1982a,b, 1983,1984; Mitchell and Kirchman 1984). In the lectin model, lectins present on the invertebrate larvae recognize a specific carbohydrate molecule from the microbial biofilm inducing

settlement. Initial experiments utilized natural sugars to block the interaction between invertebrates and surface biofilms (Kirchman and Mitchell 1983). Lectin-mediated processes can be inhibited by low molecular-weight sugars, since the added sugar competes for the carbohydrate-binding site on the protein (Sharon and Lis 1972). 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 (Kirchman et al. 1982a). However no such studies are reported in case of barnacles.

Cypris, the terminal larval instar, prolong their larval duration until a conducive substratum is available for settlement and subsequent metamorphosis. The influence of different cues is dependent on cypris larvae that have been raised through traditional rearing protocol and preconditioned at 5° C for a day or two prior to settlement assays. Holm (1990) also indicated that temporal variation in cyprid behavior may be a result of changes in larval culture conditions and/or maternal effects. Cyprids like other invertebrate larvae derive their energy from stored lipids. These competent larvae delay metamorphosis in the absence of stimuli. However, after postponement, larvae will settle in a less discriminatory manner (Rittschof et al. 1984) possibly because of depleting energy reserves (Lucas et al. 1979), which will jeopardize post metamorphic growth and/or survival (Pechenik and Cerulli 1991; Pechenik et

al. 1993). This signifies the importance of energy reserves and/ or nutritional stress of the larvae that is used.

Taking into consideration the above, following were addressed to study the cues that regulate metamorphosis of the larvae of *B. amphitrite*.

- **Significance of sugars in exploration and metamorphosis.**
- **Evaluation of different inducers from microorganisms that influence the metamorphosis.**
- **Lectins as probes to evaluate the role of signaling molecules from the bacteria.**
- **Influence of larval rearing conditions and ageing on the energetics and metamorphosis.**