J. Exp. Mar. Biol. Ecol., 156 (1992) 239–252

JEMBE 01730

Interactive effects of salinity and adult extract upon settlement of the estuarine barnacle *Balanus improvisus* (Darwin, 1854)

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(Received 13 May 1991; revision received 24 October 1991; accepted 11 November 1991)

Abstract: *Balanus improvisus* (Darwin, 1854) is distributed in mesohaline regions of estuaries worldwide. In Chesapeake Bay, USA, it occurs intermediately along a horizontal salinity gradient between two congeners, oligohaline *B. subalbidus* (Henry, 1973) and the more polyhaline *B. eburneus* (Gould, 1841). Laboratory reared cyprids of *B. improvisus* were exposed to slate substrata treated or untreated with conspecific settlement factor as salinity was varied. Peak settlement occurred at salinities of 10 and 15‰ on treated substrata, reflecting an interactive effect of these two parameters. Settlement on untreated substrata was low and showed no salinity pattern. Cyprids of *B. improvisus* settled in significantly greater numbers on intra- than on interspecific settlement factor adsorbed to slate, and they settled in lowest numbers on settlement factor from *B. eburneus* compared to *B. subalbidus*. In addition, surface effects upon settlement were less obvious as age of cyprids increased.

Key words: *Balanus improvisus*; Barnacle; Estuary; Larval settlement; Salinity; Settlement factor

INTRODUCTION

Mechanisms of settlement and metamorphosis of marine invertebrate larvae have come under intense investigation (reviews by Hadfield, 1986, and Pawlik & Hadfield, 1990). Although individual taxa vary widely in response to chemical and physical environmental stimuli, marine larval settlement and metamorphosis is generally thought to be a series of events, most likely under nervous control (Hadfield, 1978; Burke, 1983; Morse, 1984; Rittschof et al., 1986b; Bonar et al., 1990) with larval behavior determining settlement site. Understanding factors governing the settlement of sessile marine invertebrates is important both in elucidating mechanisms determining species distribution as well as in having practical implications for biofouling research.

Numerous factors have been shown to influence larval settlement and ultimately recruitment and distribution of adult barnacles. These include: relative density and location of larvae in the water column (Grosberg, 1982; Connell, 1985; Gaines et al.,

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1985; Shanks, 1986); physical attributes, such as light, flow velocity and spatial heterogeneity of potential settlement sites (Crisp, 1955; Rittschof et al., 1984; Wethey, 1984; Chabot & Bourget, 1988; Eckman et al., 1990); cyprid age (Crisp & Meadows, 1963; Branscomb & Rittschof, 1984; Rittschof et al., 1984a; Maki et al., 1988, 1990); and chemical cues, both stimulatory and inhibitory, associated with adult and larval barnacles, symbiotic fauna, flora, microbial flora and potential predators (Knight-Jones, 1953; Crisp & Meadows, 1962, 1963; Larman & Gabbott, 1975; Moyse & Hui, 1981; Strathmann et al., 1981; Rittschof et al., 1984a,b, 1985, 1986a; Standing et al., 1984; Rittschof, 1985; Yule & Walker, 1985; Raimondi, 1988; Johnson & Strathmann, 1989; Maki et al., 1988, 1990; Crisp, 1990).

Pioneering studies of gregarious settlement in *Semibalanus balanoides* (= *Balanus balanoides*) (Linnaeus, 1767) showed that settlement factor extracted from adult integument ("arthropodin"), when adsorbed to a surface, would elicit gregarious settlement in larval conspecifics (Crisp & Meadows, 1962,1963). The cyprid antennule appeared to respond to a specific molecular configuration of a protein termed "settlement factor" (Crisp & Meadows, 1963). Proteinaceous extracts which actively induced settlement were subsequently isolated and purified (review by Gabbott & Larman, 1987). The complexity of chemical mediation of barnacle larval settlement is becoming increasingly apparent in *Balanus amphitrite amphitrite* (Darwin, 1854) (Rittschof et al., 1984a, 1986b; Rittschof, 1985). For instance, settlement of *B. amphitrite* was thought to be both stimulated and inhibited by different "molecular domains" of the same bacterial exopolymers influenced by surface wettability, underscoring the complex interaction of surface, bacteria and larva (Maki et al., 1990). The interaction of environmental stimuli should be a high priority in future larval research (Hadfield, 1986; Pawlik et al., 1991).

In Chesapeake Bay, three species of *Balanus*, *B. subalbidus*, *B. improvisus* and *B. eburneus*, are distributed along an increasing horizontal salinity gradient, although considerable overlap may occur (Gordon, 1969; Poirrier & Partridge, 1979; Kennedy & DiCosimo, 1983; Dineen, pers. obs.). Given the larval retention mechanisms displayed by *B. improvisus* in the moderately stratified Miramichi Estuary (Bousfield, 1955), and similar circulation patterns in Chesaspeake Bay (Pritchard, 1952), we initiated multifactorial larval settlement studies of *B. improvisus* varying salinity, chemical cues and cyprid age because these larvae could be exposed simultaneously to varying osmotic signals as well as conspecific and congeneric adult chemical cues at some time during their planktonic existence.

Materials and Methods

ADULT BARNACLES

Adult *B. improvisus* were obtained from settlement plates or scraped from pilings either at the Smithsonian Environmental Research Center dock, Rhode River, Edgewater, Maryland (seasonal salinity range 5–15‰), or from the Chesapeake Biological Laboratory bulk heading, Solomons, Maryland (seasonal salinity range 9–14‰, 1989 – present). If not ovigerous at the time of sampling, adult *B. improvisus* were placed into culture at 12‰ salinity at 25 °C on a 15 : 9 L : D photoperiod, fed ad lib with larval *Artemia salina* and phytoplankton occasionally. Under these conditions, barnacles became ovigerous in ≈ 3 wk, and produced larvae out of the normal breeding season for settlement assays.

Adult *B. subalbidus* and *B. eburneus*, used to extract congeneric settlement factor, were obtained respectively from settlement plates deployed at Muddy Creek, the chief fresh water tributary of the Rhode River and at the Virginia Institute of Marine Science dock, York River, Gloucester Point, Virginia. Species were identified primarily from tergal plate morphology (Henry & Mclaughlin, 1975; Zullo, 1979).

LARVAE

B. improvisus larvae were obtained in one of several ways. First stage nauplii sometimes were released spontaneously from cultured adults or released by crushing naturally ovigerous barnacles. More often, paired egg masses (lamellae) were removed from ovigerous adults (Freiberger & Cologer, 1966) and placed into culture in 400 ml of 0.1-μm filter-sterilized river water. Water was changed daily and depending on the initial ripeness of the egg masses, first stage nauplii would hatch in 2–3 days. This later method was preferred because it minimized the chance of protozoan contamination when nauplii were placed into culture. Positively phototactic first stage nauplii were concentrated using a narrow light beam, and collected by pipet.

LARVAL CULTURES

≈ 10 000 first stage nauplii were placed into 4 l of aerated, 0.1-μm filter-sterilized river water at 15‰. 1 l each of a dense culture of *Phaeodactylum tricornutum* and *Isochrysis galbana* (Tahitian strain) was added initially and 400 ml each was added daily thereafter. Phytoplankton was grown in 1-l batch culture in river water that was adjusted to 15‰ salinity with Instant Ocean, 0.1-μm filter-sterilized, autoclaved and enriched with f/2 nutrient (without Tris buffer). Cultures were placed at 25 °C on a 15 : 9 h light:dark photoperiod and grown to sufficient density in 10–12 days. Antibiotics, sodium penicillin G and streptomycin sulphate (Sigma Chemical) were added to larval cultures at concentrations of 21.9 and 36.5 $mg \cdot l^{-1}$, respectively. Cyprids appeared in 6–8 days and were filtered from the culture using a 200-μm nylon sieve.

SETTLEMENT FACTOR AND SUBSTRATUM

Settlement factor was prepared similarly to that described in Rittschof et al. (1984a). Briefly, whole barnacles were extracted with distilled water, the aqueous fraction centrifuged, boiled and the resulting light yellow liquid was adjusted to a concentration of 50 $mg\ protein \cdot ml^{-1}$. Aliquots of settlement factor were frozen until immediately before use.

Slate substrata, 3 × 6.5 cm, were initially scrubbed under running tap water and thoroughly rinsed in distilled water. Prior to settlement assays, all substrata were submerged in coarsely filtered, aerated, 15‰ estuarine water for 48 h. Immediately before use, substrata were air-dried and either placed into culture dishes untreated (control) or adsorbed with settlement factor as follows. Using a camel hair brush, settlement factor was thrice painted evenly on the slate, and gently air dried after each application. Care was taken at all times to use gloves while handling substrata.

SETTLEMENT ASSAY PROCEDURES

Routinely, 200 cyprids were pipetted into paraffin-coated, disposable polystyrene dishes (4 cm deep, 10 cm in diameter), containing slate substrata either treated or untreated with settlement factor. Salinity was subsequently adjusted slowly with distilled water or a saline (Instant Ocean) solution of 40‰ to attain salinity treatments of 2, 5, 10, 15, 20, 25, 30 or 35‰. Each of the 16 treatment combinations (+ or − settlement factor × 8 salinities) were replicated twice for larvae obtained from naturally ovigerous adult barnacles and twice for larvae obtained from barnacles induced to breed in the laboratory. Salinity adjustment did not appear to adversely affect the normal exploratory behavior of the cyprid in any treatment. Test dishes were then examined for metamorphosed cyprids (juvenile barnacles) at 24-h intervals up to 144 h.

Experiments testing relative settlement enhancement by conspecific vs. congeneric extract were run on slate substrata at 10‰ salinity, using 100 cyprids $\cdot$ trial^{-1}, with four replicates.

Experiments investigating settlement behavior of aged cyprids were run as follows. Cyprids from the same mass culture were divided approximately equally into two groups and placed in the dark at 5 °C. Under these conditions, cyprids do not swim or settle on container walls (Rittschof et al., 1984a). One group was placed into settlement culture the following day, Day 1, and the second group was "aged" under these conditions until Day 8. Settlement of the two groups was assayed on slate substrata, in the presence of settlement factor only, at a salinity of 10‰ with 100 cyprids $\cdot$ trial^{-1}, three replicates per treatment.

DATA ANALYSES

All data were treated as categorical. Experiments investigating the effects of salinity, settlement factor, and their interaction on settlement frequency were analyzed using logistic regression (PROC CATMOD with maximum likelihood estimation, 0.5 added to all cells; SAS Institute, 1985). Logistic regression was also used to determine whether the frequency of larval settlement was affected by salinity, cyprid age or their interaction. Two-way contingency tables were used to compare settlement frequency and different types of settlement factor. Unplanned multiple comparisons, controlling for experiment-wise Type I error, were used to distinguish among frequencies (simultaneous test procedures (STP), Sokal & Rohlf, 1981, p. 728).

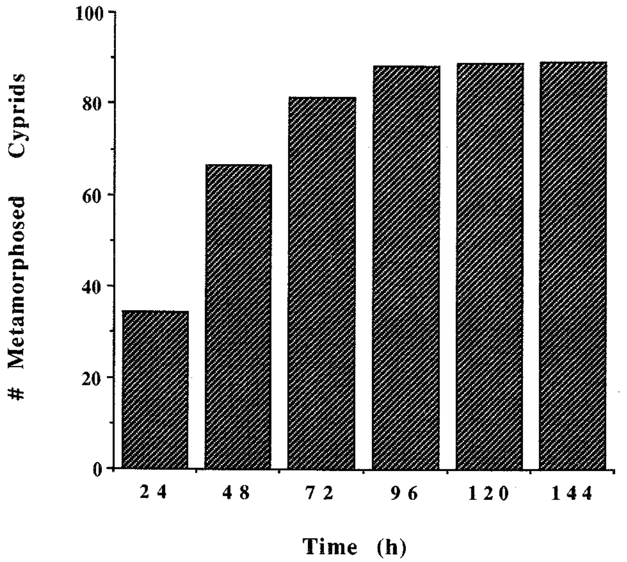

Fig. 1. Cumulative larval settlement of *B. improvisus* at 24-h intervals exposure to slate substrata treated with conspecific settlement factor at a salinity of 10‰. Averages of two replicate assays with 200 cyprids · trial^{-1} are shown.

All data are presented at 144 h, after which time, no appreciable settlement occurred (see Fig. 1).

RESULTS

SALINITY–SETTLEMENT FACTOR INTERACTION

Settlement frequencies of *B. improvisus* cyprids obtained from naturally ovigerous barnacles exhibited significant interactive effects of salinity and settlement factor (log linear model, $\chi^2 = 106.6$, 7 df, $p < 0.001$) (Fig. 2). At every salinity except 2 and 35‰, settlement frequency was significantly higher in the presence of settlement factor than in its absence (pairwise *G* tests; $p < 0.05$, experimentwise error rate = 0.05, 7, 1 df, $\chi^2 = 7.2$). Settlement frequency in the presence of settlement factor did not differ significantly at either 2 or 35‰ salinity. Settlement frequencies at 5 and 10‰ salinity were significantly higher than at all other salinity levels in the presence of settlement factor (unplanned multiple comparisons, STP tests; $\chi^2 = 18.5$, 7 df, $p < 0.01$).

When *B. improvisus* cyprids were obtained from barnacles induced to breed in the laboratory, settlement frequencies again exhibited significant interactive effects of salinity and settlement factor (log linear model, $\chi^2 = 20.9$, 7 df, $p < 0.004$) (Fig. 3). Settlement frequency was also highly dependent on the presence of settlement factor and showed significant differences at every level except 35‰ (pairwise *G* tests; $p < 0.05$,

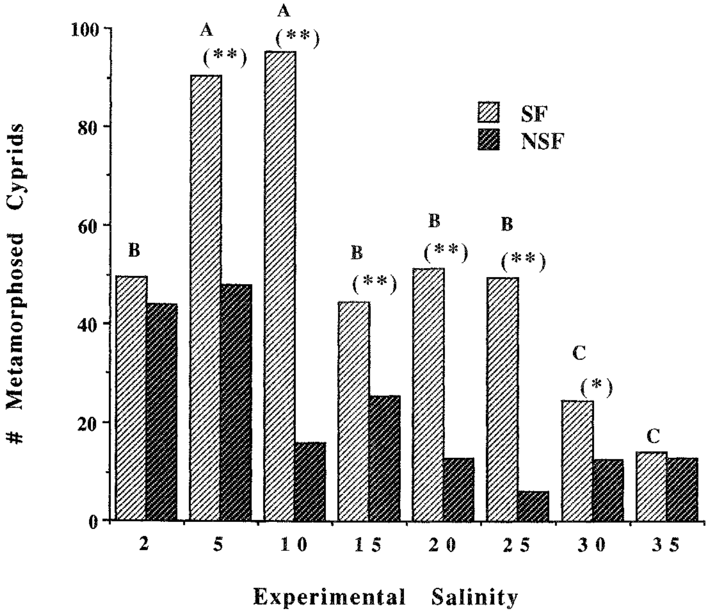

Fig. 2. Average settlement of *B. improvisus*, when larvae were obtained from naturally ovigerous adults, showing effect of treated (SF) and untreated (NSF) slate substrata with settlement factor at various salinities. For settlement factor treatments only, settlement frequencies at salinities with same letter were not significantly different (STP tests, $p < 0.01$). Significant differences in settlement between settlement factor treatments at each salinity level are designated as: $^*p < 0.05$, $^{**}p < 0.01$. Two replicate assays were performed per treatment with 200 cyprids · trial^{-1}.

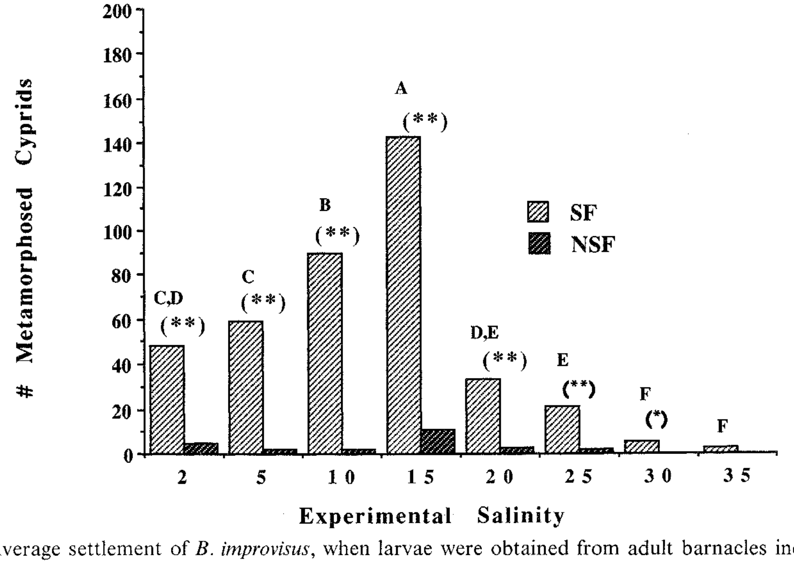

Fig. 3. Average settlement of *B. improvisus*, when larvae were obtained from adult barnacles induced to breed in the laboratory, showing effect of treated (SF) and untreated (NSF) slate substrata with settlement factor at various salinities. For settlement factor treatments only, settlement frequencies at salinities with same letter were not significantly different (STP tests, $p < 0.01$). Significant differences in settlement between settlement factor treatments at each salinity level are designated as: $^*p < 0.05$, $^{**}p < 0.01$. Two replicate assays were performed with 200 cyprids · trial^{-1}.

experimentwise error rate = 0.05, 7, 1 df, $\chi^2 = 7.2$). Cyprid settlement, in the presence of settlement factor, was significantly higher at 15‰ salinity than at any other level (unplanned multiple comparisons, STP tests; $\chi^2 = 18.5$, 7 df, $p < 0.01$).

CONSPECIFIC AND CONGENERIC SETTLEMENT FACTOR

B. improvisus settlement frequency was significantly affected by type of settlement factor adsorbed to slate substrata ($\chi^2 = 422.3$, 4 df, $p < 0.001$) (Fig. 4). *B. improvisus* settlement frequencies were greatest in the presence of conspecific settlement factor, followed in decreasing order by settlement factor extracted from *B. subalbidus*, *B. eburneus* at 10‰ salinity, and *B. eburneus* at 25‰ salinity (unplanned multiple comparisons, STP tests; $\chi^2 = 13.3$, 4 df, $p < 0.01$).

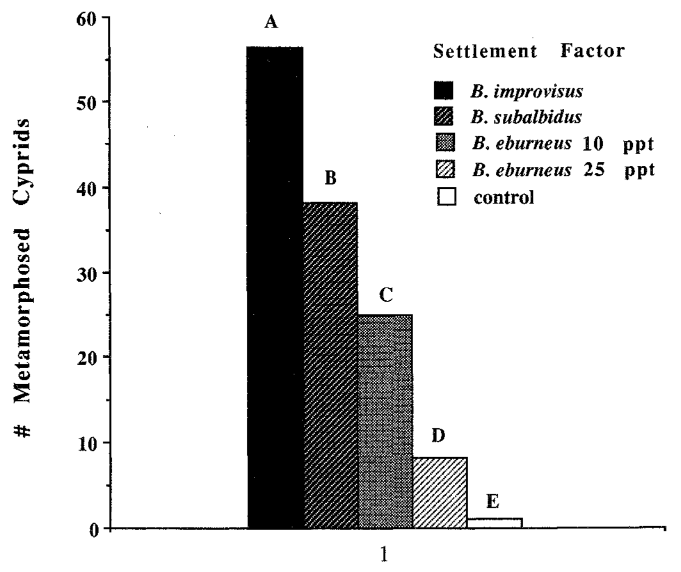

Fig. 4. Average settlement of *B. improvisus*. Effect of settlement factor prepared from *B. improvisus* and *B. subalbidus* and no settlement factor (control) at an assay salinity of 10‰ and settlement factor prepared from *B. eburneus* at an assay salinity of 10 and 25‰ on slate. Letters designate significant differences in settlement frequency among treatments, $p < 0.01$. Four replicate assays were performed for each treatment with 100 cyprids · trial^{-1}.

AGING OF CYPRIDS

Settlement frequencies of young and aged cyprids of *B. improvisus* were not the same across all salinities ($\chi^2 = 75.8$, 7 df, $p < 0.001$) in the presence of settlement factor. Young cyprids settled in higher frequencies than older cyprids at 5 and 10‰ salinity. Settlement frequency did not differ between young and old cyprids at 15‰ salinity. Aged cyprids settled at significantly higher frequencies than younger cyprids at 20, 25 and

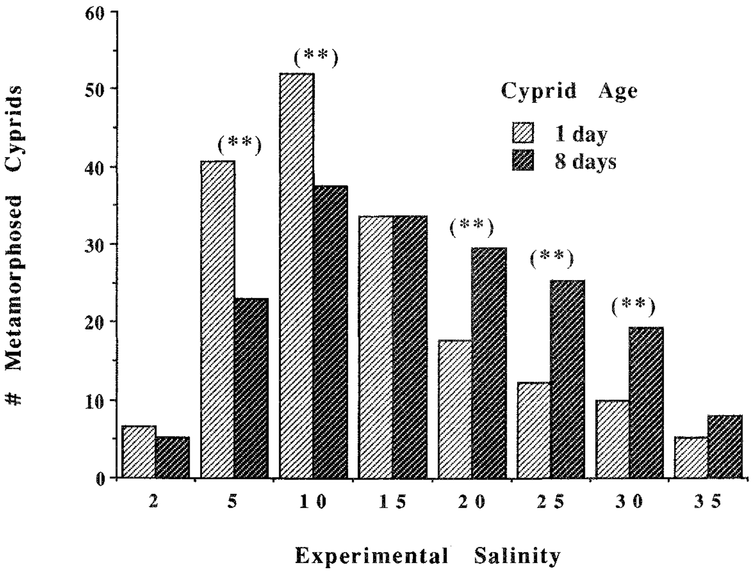

Fig. 5. Average settlement of *B. improvisus* cyprids from same larval cohort aged for 1 and 8 days. Settlement was assayed on slate substrata treated with conspecific settlement factor. Significant differences in settlement frequency between settlement factor treatments at each salinity are designated as: $^{**}p < 0.01$. Three replicate assays were performed with 100 cyprids · trial^{-1}.

30‰ salinity (pairwise *G* tests; $p < 0.01$, experimentwise error rate = 0.05, 7, 1 df, $\chi^2 = 10.2$). No significant differences in settlement frequency between young and aged cyprids occurred at either 2 or 35‰ salinity (Fig. 5). Overall settlement in the presence of settlement factor, disregarding salinity treatments, was similar in both young and aged cyprids from the same larval cohort, 22.3 and 22.7% respectively.

Discussion

Salinity is one of the major physical factors determining the distribution of benthic organisms in estuaries (Boesch, 1977). Distribution of sessile estuarine organisms along a salinity gradient could be due either to: (1) preferential larval settlement at, for example, a salinity regime appropriate to the adult phase, which in turn could be the result of a specific larval retention mechanism allowing the planktonic larva to remain in or return to a particular location; and/or (2) juvenile die-off following indiscriminate settlement (Keough & Downes, 1982; Connell, 1985). *B. improvisus* is distributed in estuaries worldwide (see Furman, 1990) and is tolerant of a wide range of salinity, an obvious adaptation to this habitat. Several reports have indicated the influence of salinity relative to adult barnacle (Turrpaena & Smikina, 1961; Gordon, 1969; Branscomb, 1976; Poirrier & Partridge, 1979; Kennedy & DiCosimo, 1983) and adult/larval barnacle distribution (Bousfield, 1955) in stratified estuaries. The later study reports adult *B. improvisus* occurring at a salinity of 0–13‰, the most landward populations of which are subject to mortality after prolonged exposure to low salinities via river discharge. In addition, demonstrating larval retention mechanisms in this estuary, naupliar stages of *B. improvisus* were found at various salinities depending on their ontogeny. The cyprid mode occurred at 17‰, which corresponded to the average summer salinity where adult *B. improvisus* was most numerous (Bousfield, 1955). Maximal larval settlement of *B. improvisus* in our experiments at mid-salinities, in the presence of conspecific settlement factor (Figs. 2, 3), agree with peak settlement implications in Bousfield's study.

INTERACTION OF SALINITY AND SETTLEMENT FACTOR PROTEIN

Planktonic larvae are exposed simultaneously to numerous environmental stimuli, many of which can act as settlement cues singly or in concert. Ionic stimulation alone can induce settlement in some invertebrate groups (Werner & Buchal, 1973; Baloun & Morse, 1984; Yool et al., 1986; Pechenik & Heyman, 1987) but our experiments, as well as others (Rittschof et al., 1986b), indicate that this is not the case for larval barnacles, because salinity variation alone did not induce appreciable settlement of *B. improvisus* cyprids (Figs. 2, 3).

In our experiments, salinity and the presence of settlement factor protein interacted to stimulate larvae to settle in greatest frequencies at mid-salinities. However, the precise nature of such an interactive stimulus to settlement is not clear and could be due to a number of complex phenomena. For instance, the degree of adsorption–desorption of (settlement factor) protein can be affected by: (1) substratum surface energy, (2) salinity and (3) protein concentration (Rittschof et al., 1984a; Crisp et al., 1985; Roberts et al., 1991). In addition, salinity can change the conformation of a protein, thereby changing the three-dimensional spatial arrangement of its native state. Two theories account mechanistically for the perception of adsorbed settlement factor protein by the cyprid antennule. One involves a chemosensory recognition of amino acid sequences of settlement factor protein brought about by an enzymatic discharge of cyprid antennular glands (Nott & Foster, 1969). Tegtmeyer & Rittschof (1989) stimulated settlement and metamorphosis of *B. amphitrite* using synthetic peptide analogs to barnacle settlement pheromone. An alternative theory likens the cyprid antennular recognition to a "tactile chemical sense" whereby surface properties of antennular cuticular hairs of the cyprid cause it to adhere to the molecular configuration of adsorbed settlement factor protein, similar to a specific antigen-antibody response. The strength of temporary cyprid adhesion was shown to increase in the presence of adsorbed settlement factor thus lending support to this second alternative (Yule & Crisp, 1983). In the present study, adsorption of settlement factor protein at various salinities was not measured and likely varied, possibly influencing degree of settlement. However, based on the antigen–antibody analogy, we propose another hypothesis that cyprids respond to a change in conformation of settlement factor protein brought about by experimental salinity. Varying salinity may render settlement factor protein either more or less attractive to the cyprid antennule to mount a specific recognition response.

The subtle differences in settlement behavior of larvae obtained from naturally ovigerous adults, as opposed to laboratory cultured adults, indicate that settlement of *B. improvisus* could be affected by the environment in which adult barnacles brood embryos. For instance, when larvae were obtained from ovigerous adults (in the spring of the year when salinities are characteristically low) as opposed to larvae obtained from adults induced to breed in the laboratory under constant culture conditions (i.e., 12‰), settlement peaked at lower salinities in the presence of settlement factor (Figs. 2, 3) and variability of mean settlement in replicate assays was relatively high in the former group, implying an acclimation effect of salinity relative to settlement on developing embryos.

SETTLEMENT AND TAXONOMIC AFFINITY

Barnacle surfaces can elicit settlement to varying degrees depending on their phylogenetic affinity (Knight-Jones, 1955; Crisp & Meadow, 1962; Crisp, 1990). In the present study, exposure of *B. improvisus* cyprids to various adult extracts adsorbed to slate substrata at an intermediate salinity produced a decreasing hierarchy of settlement in response to settlement factor from: (1) conspecifics, (2) *B. subalbidus* and (3) *B. eburneus* (Fig. 4). Using discriminant function analyses of the "*B. amphitrite* complex" that includes *B. amphitrite*, *B. improvisus*, *B. eburneus* and *B. subalbidus* (among others), Henry & Mclaughlin (1975) suggest that *B. improvisus* is more nearly allied to *B. eburneus* than to *B. subalbidus*. The apparent contradiction between settlement levels seen in our study and phylogenetic affinity suggest that ecological factors may also be important. Larvae of *B. improvisus* subject to estuarine circulation (Bousfield, 1955) could be exposed to adult cues from these congeners, particularly in areas of overlapping distribution. Extrapolating to field situations, by settling near-oligohaline *B. subalbidus* rather than near-polyhaline *B. eburneus*, *B. improvisus* would distribute in lower-saline waters nearer the head of the estuary increasing the likelihood of its larvae being retained within the estuary. Cyprids of *B. subalbidus* also showed a greater tendency to settle in the presence of *B. improvisus* than in the presence of *B. eburneus* extract (Dineen & Hines, unpubl. data).

Using similar ecological reasoning that, since *B. eburneus* is characteristically located in higher-saline waters, we tested if settlement factor from *B. eburneus* would be more stimulatory to cyprids of *B. improvisus* at higher salinities than at lower salinities. When *B. improvisus* cyprids were exposed to *B. eburneus* settlement factor under these conditions, ≈2/3 less settlement occurred at the higher salinity (Fig. 4). Again, this difference in settlement frequency could reflect experimental salinity affecting degree of adsorption and/or a change in conformation of *B. eburneus* settlement factor protein making it less stimulatory to *B. improvisus* cyprids at higher salinities.

SETTLING BEHAVIOR OF OLDER CYPRIDS

An increased rate of settlement with age in cyprids was demonstrated as early as 1963 with *S. balanoides* (Crisp & Meadows, 1963). Loss of discriminating settlement behavior in aged cyprids of *B. amphitrite* occurs in the presence of low-frequency sound waves (Branscomb & Rittschof, 1984), polystyrene (Rittschof et al., 1984a) and bacterial films (Maki et al., 1988); although it was later shown that some bacteria are capable of inhibiting settlement of older cyprid larvae (Maki et al., 1990).

In the present study, overall settlement levels of *B. improvisus* were strikingly similar in both young and old cyprids from the same larval cohort. And, even though peak settlement for both young and aged cyprids occurred at the same salinity, peak levels were higher for young cyprids than older cyprids and more young cyprids settled at lower salinities. In contrast, aged and perhaps less discriminating cyprids settled more at higher-salinity regimes. This shift could imply that older cyprids, with presumably spent energy reserves (Lucas et al., 1979), could settle in the presence of established adult populations in higher-saline waters rather than not settle at all. However, in interpreting these results, it should be kept in mind that artificially aging cyprids in the cold, as was done in our experiments and in other studies, is probably not equivalent to aging larvae at higher temperatures.

Interactions between settlement factor and salinity could have profound implications for structuring barnacle populations in estuaries. By showing peak settlement at 10–15‰ cyprids of *B. improvisus* distribute in mid-estuarine zones likely avoiding adult physiological stress at salinity extremes. By showing higher settlement in the presence of *B. subalbidus* as opposed to *B. eburneus* settlement factor, *B. improvisus* would likely settle in lower-salinity areas near the head of the estuary, thus increasing the likelihood of its larvae being retained within the estuary. And finally, settlement at higher-salinity regimes by older cyprids could reflect the ability of *B. improvisus* larvae to take advantage of a last chance to settle in the estuary before being flushed from it.

ACKNOWLEDGEMENTS

We gratefully acknowledge N. Targett for thought-provoking comments during initiation of research. D. Rittschof and A. Schmidt provided invaluable information regarding larval rearing techniques. G. Baptist generously provided algal inoculums and nutrient. We thank D. Henry and V. Zullo for species identifications. Finally, we appreciate the statistical assistance from L. D. Smith and comments on the manuscript from two anonymous reviewers. Research was, in part, supported by a Smithsonian Postdoctoral Fellowship to J. F. Dineen, Jr., and a grant from the Smithsonian Environmental Sciences Program to A.H. Hines.

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