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Larval settlement of the polyhaline barnacle *Balanus eburneus* (Gould): cue interactions and comparisons with two estuarine congeners

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Abstract

The effect of salinity on larval settlement of the polyhaline estuarine barnacle *Balanus eburneus* (Gould) was investigated by challenging laboratory reared cyprids to settle (permanently attach and metamorphose) under a number of permutations of salinity and adult extract (settlement factor). When cyprids were exposed to an array of salinities ranging from 2 to 35 ppt in the presence of conspecific extract adsorbed to slate panels, maximum settlement occurred at salinities of 15 and 20 ppt. In the absence of adult extract, few larvae settled at any salinity. No differences in settlement frequencies across the array of test salinities were observed between replicate aliquots within a cyprid batch, but significant differences in settlement were observed between batches of cyprids. When settlement of newly molted cyprids was contrasted with that of cyprids forced to postpone metamorphosis, settlement peaked at 20 and 15 ppt salinity, respectively, and overall settlement levels of the "delayed" cohort increased. *B. eburneus* cyprids settled significantly more frequently on substrata adsorbed with conspecific extract than on substrata adsorbed with extract from *B. improvisus* (Darwin) or *B. subalbidus* (Henry), but settlement on these latter two treatments did not differ. Although post-settlement processes cannot be overlooked, our results indicate that larval behavior at settlement could play a substantial role in determining adult distribution of *B. eburneus* along the estuarine salinity gradient. Contrasting settlement patterns of *B. eburneus* with those obtained previously for mesohaline *B. improvisus* and oligohaline *B. subalbidus* indicates that settlement behavior of the former two species could be influential in structuring their distribution, whereas pre-settlement behavior could be a determining factor in *B. subalbidus* distribution.

Key words: Adult extract; *Balanus eburneus*; Barnacle; Estuary; Settlement; Salinity

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1. Introduction

Planktonic larvae of estuarine invertebrates display a variety of behavioral mechanisms thought to enhance retention within, or return to the estuary in order to settle in appropriate habitat. For example, variation in vertical position in the water column during larval development facilitates retention of barnacle (Bousfield, 1955) and other invertebrate larvae (Carriker, 1967; Sandifer, 1975; Cronin, 1982; Sulkin, 1984; Epifanio, 1988) in stratified estuaries. On a local scale, a cohort of cypris larvae could be transported to an estuarine area where salinity and/or specificity of chemical cues from previously established adult populations differ. Gregarious cypris settlement (Knight-Jones & Stevenson, 1950), whether defined as avoidance of unsuitable substrata (Hui & Moyse, 1987) or more traditionally as aggregative settlement on suitable substrata, e.g. in the presence of adsorbed conspecific adult extract (Crisp & Meadows, 1963), occurs in estuaries to varying degrees (Bousfield, 1955; Gordon, 1969; Bacon, 1971; Poirrier & Partridge, 1979; Kennedy & DiCosimo, 1983). Since post-larval settlement is especially critical for larvae of sessile adults in a fluctuating environment such as an estuary, understanding factors influencing settlement are an essential step in understanding adult distribution.

We have conducted laboratory experiments on the interactive effects of salinity and adult extract (settlement factor) on larval settlement of three cosmopolitan estuarine barnacles: *Balanus eburneus* (Gould); *B. improvisus* (Darwin); and *B. subalbidus* (Henry). These congeners are sequentially distributed along a horizontal salinity gradient in Chesapeake Bay and many other estuaries, although considerable overlap does occur. *B. eburneus* has been reported from meso- to polyhaline salinity regimes in Chesapeake Bay (8.5–18 ppt) (Gordon, 1969; Kennedy & DiCosimo, 1983) and in Lake Ponchartrain, Louisiana (12–19 ppt) (Poirrier & Partridge, 1979), and from oligo-to polyhaline regimes in Trinidad, West Indies (5–30 ppt) (Bacon, 1971). *B. improvisus* occurs most abundantly in mesohaline regimes of the Miramichi (Bousfield, 1955) and British estuaries (Furman & Yule, 1989), as well as in the Chesapeake (Gordon, 1969; Kennedy & DiCosimo, 1983; Dineen & Hines, 1992) and San Francisco (Newman, 1965) Bays and in Lake Ponchartrain (Poirrier & Partridge,1979). *Balanus subalbidus* predominates in low saline head waters of Chesapeake Bay (Kennedy & DiCosimo, 1983; Dineen & Hines,1994) and is able to withstand prolonged exposure to fresh water (Poirrier & Partridge, 1979; Dineen, pers. obs.). The occurrence of all three cosmopolitan barnacle species along the estuarine gradient in Chesapeake Bay has presented a unique opportunity to assess and compare the degree to which larval settlement (defined as permanent attachment and metamorphosis) could be contributing to this differential distribution.

Larval settlement of *B. improvisus* and *B. subalbidus* differ in their interactive response to salinity and adult extract (Dineen & Hines, 1992, 1994). In the laboratory, *B. improvisus* settles at mid-salinities (10–15 ppt) in the presence of conspecific settlement factor, coinciding with its mesohaline adult distribution in the field (Dineen & Hines, 1992). *Balanus subalbidus* however, not only settles in the laboratory at low salinities, but settles at much higher salinities than the oligohaline distribution of adults in the estuary would indicate (Dineen & Hines, 1994). In addition, while larvae of both congeners can distinguish conspecific from congeneric extract, delaying metamorphosis results in less discriminating settlement relative to salinity only in *B. improvisus*. We present here results of a third series of similar experiments testing larval settlement responses of polyhaline *B. eburneus* to three interactive variables: (1) adult extract, known to stimulate gregarious larval settlement particularly in conspecific barnacles (Crisp & Meadows, 1962); (2) salinity, since ionic cues are known to stimulate settlement/metamorphosis in some invertebrates (Baloun & Morse, 1984), although not in barnacles (Rittschof et al., 1986); and (3) metamorphic delay, known to influence settlement discrimination (Rittschof et al., 1984). Results of this complemental series of experiments allow us to contrast settlement patterns of all three estuarine species.

2. Materials and methods

2.1. *Adult and larval barnacles*

Adults of *B. eburneus* used as a source either for larvae or for adult extract were obtained from lucite settlement plates suspended from the Virginia Institute of Marine Science dock, York River, Gloucester Point, Virginia. By increasing food and temperature, adult barnacles were induced to breed aseasonally in the laboratory. *Balanus eburneus* cyprids used in settlement assays were obtained from mass culturing nauplii which hatched from lamellae dissected from brooding adults (Freiberger & Cologer, 1966; Dineen & Hines, 1992).

2.2. *Experimental procedure*

Slate panels (3 × 5 cm) adsorbed with adult extract (see below) or untreated, were used as experimental substrata and placed in individual 10 cm diameter disposable polystyrene dishes containing 0.1 μm filtered estuarine water. *Balanus eburneus* does not attach to polystyrene (Dineen & Hines, unpubl.). One hundred cyprids were subsequently introduced to each dish, and metamorphosed individuals (i.e. juvenile barnacles) were counted every 24 h for 5 or 6 days. Data for metamorphic delay experiments are presented at 120 h; all other data are presented at 144 h.

Two different batches of cyprids were used to examine the combined effects of salinity and adult extract on metamorphosis. One of these larval batches was divided into two replicate aliquots. Experiments were conducted at 8 salinities (2, 5, 10, 15, 20, 25, 30, 35 ppt) in the presence and absence of conspecific adult extract. For both batches of larvae (including the batch with replicate aliquots), cyprids were partitioned into 16 treatments (i.e. 8 salinities, with and without settlement factor). Each of the 48 trials (16 treatments × 3 batch/aliquots) received 100 cyprids. The salinity × settlement factor treatments were compared within and between batches using log-linear models described below, comparing main effects of replicate/batch and treatment as well as interaction effects.

Balanus eburneus cyprids used in settlement experiments testing effect of metamorphic delay on settlement were obtained by halving the same larval cohort: the first group (untreated) was placed into assay dishes approximately 24 h after molting to the cyprid; the second group ("delayed") was first placed in the dark at 5 °C for 7 days (Rittschof et al., 1984). Settlement in both groups was assayed in the presence of settlement factor only at 8 salinities as above. Treatments were replicated three times.

Experiments comparing the effect of conspecific (*B. eburneus*) and congeneric (*B. improvisus* and *B. subalbidus*) adult extract on metamorphosis were run as above, on slate substrata adsorbed with appropriate extract, at 20 ppt salinity only, 100 cyprids per trial, and replicated three times.

2.3. *Adult extract (settlement factor)*

Whole adult barnacles (*B. eburneus*) were crushed in distilled water. Resulting liquid was decanted, centrifuged, boiled and frozen until immediately before use. It was applied to slate substrata at a concentration of 50 μg protein/ml (Pierce Bioassay) (Rittschof et al., 1984; Dineen & Hines, 1992). Congeneric settlement factor was extracted similarly from adults of two species: *B. improvisus* sampled from the Rhode or Patuxent Rivers, central Chesapeake Bay, Maryland, and *B. subalbidus*, obtained from either the Baltimore Harbor area (upper Chesapeake Bay, Maryland), or Muddy Creek, the chief fresh water tributary of the Rhode River.

2.4. *Data analyses*

Settlement frequencies in experiments investigating adult extract and salinity or metamorphic delay and salinity, and their interaction, were analyzed using logistic regression (PROC CATMOD with maximum likelihood estimation, SAS Institute, 1985). Contingency analysis was used to compare settlement frequencies in the presence of conspecific and congeneric extract followed by unplanned multiple comparisons controlling for experimentwise Type 1 error to distinguish among frequencies [Simultaneous Test Procedures (STP), Sokal & Rohlf, 1981].

3. Results

Cyprid mortality in all experimental trials was low, i.e. usually below 10% when experiments were terminated at either 120 or 144 h. Mortality showed no pattern relative to experimental salinity, source of settlement factor, or treatment of cyprid. Consequently, we report results in terms of number of cyprids which metamorphosed to juvenile barnacles from the initial allotment of 100 cyprids per trial.

Replicate aliquots of cyprids from the same larval batch showed no differences in settlement across the array of test salinities in the presence and absence of settlement factor ($\chi^2 = 0.17$, 1 df, $p > 0.7$), nor were there significant interactive effects of replicate × treatment ($\chi^2 = 11.6$, 7 df, $p > 0.1$). Significant differences in settlement frequencies were observed between batches of cyprids ($\chi^2 = 14.9$, 1 df, $p < 0.001$), but there were no interaction effects of batch × treatment ($\chi^2 = 4.9$, 7 df, $p > 0.7$). Because of the overall similarity in settlement pattern, we averaged settlement frequencies between the two batches of cyprids. Experiments investigating the influence of salinity and the presence and absence of conspecific adult extract on *B. eburneus* settlement resulted in significant interactive effects (log linear model, $\chi^2 = 20.4$, 7 df, $p < 0.005$) (Fig. 1). Markedly more settlement occurred in the treatments with settlement factor than those without, and at each salinity, significantly more larvae metamorphosed in the presence of conspecific settlement factor than on untreated substrata (pairwise *G*-tests; $p < 0.01$, experimentwise error rate = 0.05, 7, 1, df, $\chi^2 = 10.2$). Significant differences in settlement frequencies across all salinities occurred both in the presence ($\chi^2 = 600.9$, 7 df, $p < 0.001$) and absence ($\chi^2 = 39.8$, 7 df, $p < 0.001$) of conspecific adult extract. Settlement frequencies in the presence of settlement factor peaked at 65–70% in salinities of 15 and 20 ppt and without settlement factor, maximum settlement frequencies of 5–8% occurred at 20–25 ppt salinity (unplanned multiple comparisons, STP tests; $\chi^2 = 15.5$, 8 df, $p < 0.05$).

Settlement of *B. eburneus* cyprids exhibited significant interactive effects of metamorphic postponement and salinity (log linear model, $\chi^2 = 36.8$, 7 df, $p < 0.001$) (Fig. 2). Settlement of *B. eburneus* differed significantly among salinities for both newly molted cyprids ($\chi^2 = 498.5$, 7 df, $p < 0.001$) and those which were forced to postpone metamorphosis ($\chi^2 = 417.7$, 7 df, $p < 0.001$). In the presence of conspecific settlement factor, newly molted *B. eburneus* cyprids settled at highest frequency at salinities of 15 and 20 ppt (unplanned multiple comparisons, STP tests; $\chi^2 = 15.1$, 5 df, $p < 0.01$); whereas settlement of the delayed cohort peaked at 15 ppt (unplanned multiple comparisons, STP tests; $\chi^2 = 16.8$, 6 df, $p < 0.01$). Overall settlement of the metamorphi-

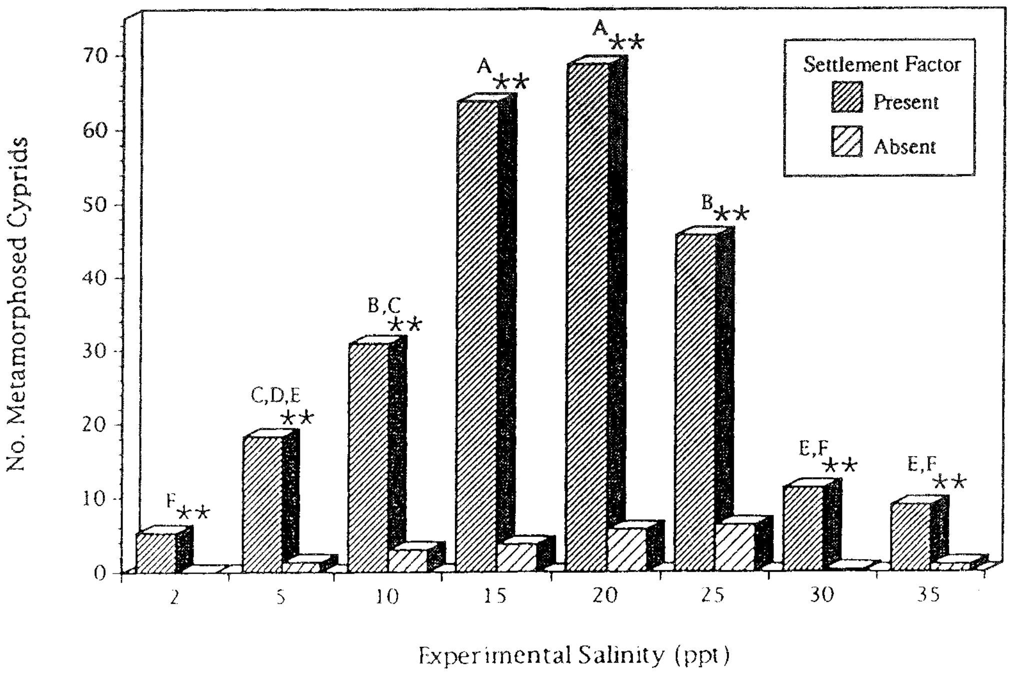

Fig. 1. Influence of salinity and settlement factor on larval attachment and metamorphosis of *B. eburneus*. Significant differences in settlement between salinities in the presence of settlement factor are designated by letters. Significant differences in settlement between settlement factor treatments at each salinity are designated by (**), $p < 0.01$. Bars indicate means of three replicate trials with 100 cyprids per trial.

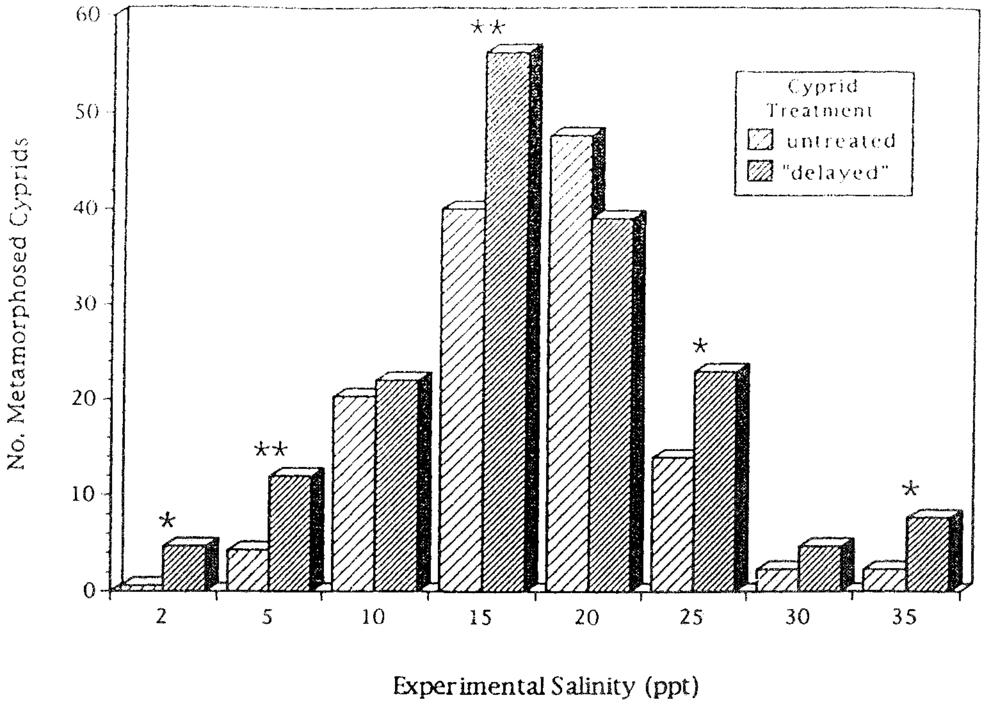

Fig. 2. Influence of metamorphic delay on larval attachment and metamorphosis of *B. eburneus*. Average number of newly molted (untreated) and metamorphically delayed ("delayed") cypris larvae metamorphosing at experimental salinities in the presence of adult settlement factor. Significant differences in settlement between larval cohorts at various salinities are designated by (**), $p<0.01$ and (*), $p<0.05$. Bars represent means of three replicate trials with 100 cyprids per trial.

cally delayed larval cohort was greater than the untreated cohort (21.2% compared to 16.4%). The former group settled at greater frequency than untreated larvae at salinities of 5, 15 (pairwise *G*-tests; $p<0.01$, experimentwise error rate = 0.05, 7, 1 df, $\chi^2 = 10.2$) 2, 25 and 35 ppt (pairwise *G*-tests; $p<0.05$, experimentwise error rate = 0.05, 7, 1 df, $\chi^2 = 7.2$).

Settlement of *B. eburneus* cyprids differed significantly among substrata adsorbed with conspecific and congeneric settlement factors ($\chi^2 = 203.9$, 3 df, $p<0.001$) (Fig. 3). Settlement was significantly greater in the presence of conspecific settlement factor, but did not differ in treatments of settlement factor extracted from congeners *B. subalbidus* and *B. improvisus* (unplanned multiple comparisons, STP tests; $\chi^2 = 9.2$, 2 df, $p<0.01$). Settlement in all treatments with adult extract was significantly greater than in controls with no extract.

4. Discussion

4.1. Congeneric distribution and settlement

Factors influencing settlement of marine invertebrate larvae are not only important in understanding the settlement process itself, but also in understanding the distribution of adult populations. Intuitively, the ability of an estuarine larva to cue into

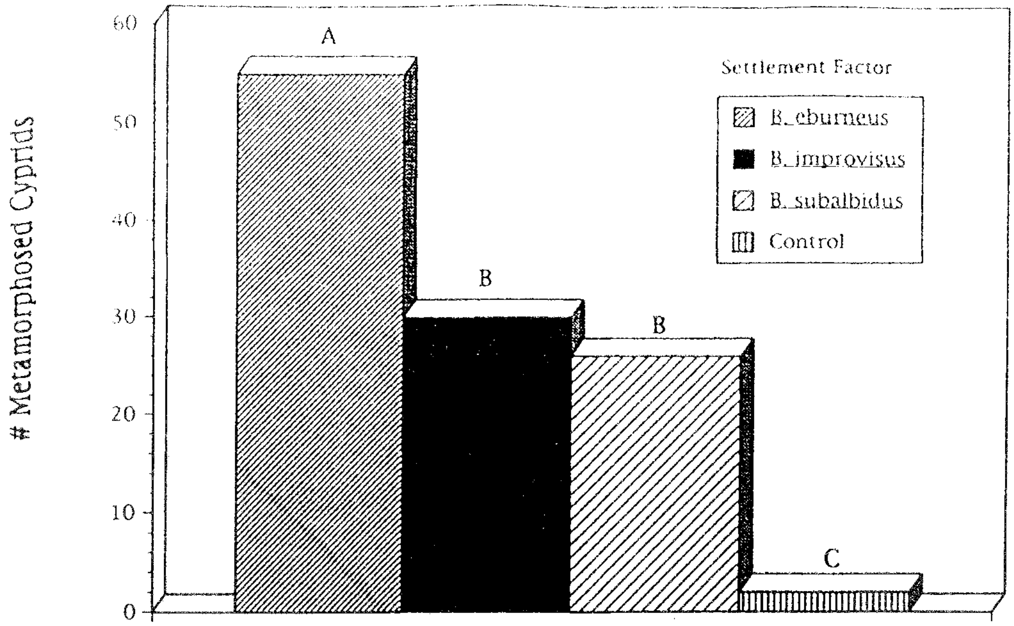

Fig. 3. Influence of source of settlement factor on larval attachment and metamorphosis of *B. eburneus*. Average number of metamorphosed cyprids in the presence of conspecific (*B. eburneus*), congeneric (*B. improvisus*, *B. subalbidus*) and no (control) settlement factor at 20 ppt salinity. Settlement factor was applied at a concentraton of 50 μg protein/ml in all cases. Significant differences in settlement are designated by letters. Bars indicate means of three replicate trials with 100 cyprids per trial.

established conspecific adult populations, regardless of ambient salinity at the time of settlement, would assure its placement in an area known to have previously supported adult growth. However, our laboratory studies of both *B. eburneus* and *B. improvisus* indicate that cyprid settlement of these species, in the presence of adult chemical cue, is each significantly enhanced at a particular salinity. For example, larval settlement responses peaked synergistically in the presence of conspecific adult settlement factor at meso-polyhaline salinities for *B. eburneus* (Figs. 1 and 4a) and at mesohaline salinities for *B. improvisus* (Dineen & Hines, 1992). For both species, salinity alone had little effect in stimulating settlement on slate substrata (Fig. 4b). This pattern suggests that larval behavior, stimulated by an interaction of salinity and adult chemical cue at the time of settlement, could be influential in determining distribution of both *B.eburneus* and *B. improvisus*.

Our studies are the first to examine the potential effects of salinity on adult extract in inducing cyprid settlement and metamorphosis of estuarine congeners. Settlement inducement properties of adult extract in larval barnacles has been well established (Gabbott & Larman, 1987). Since cypris larvae metamorphose in the presence of adult chemical cues only when these cues are adsorbed to an appropriate surface (Crisp & Meadows, 1963; Rittschof, 1985), and, if we assume that in our experiments adult extract was applied in sufficient quantity to offset dilution effects (see Crisp, 1990), then our results suggest that peak settlement of *B. eburneus* and *B. improvisus* at their respective salinity regimes reflect either a more attractive surface or an enhanced larval perception of settlement cue at these salinities.

Larval settlement of *B. subalbidus*, on the other hand, appears less dependent on

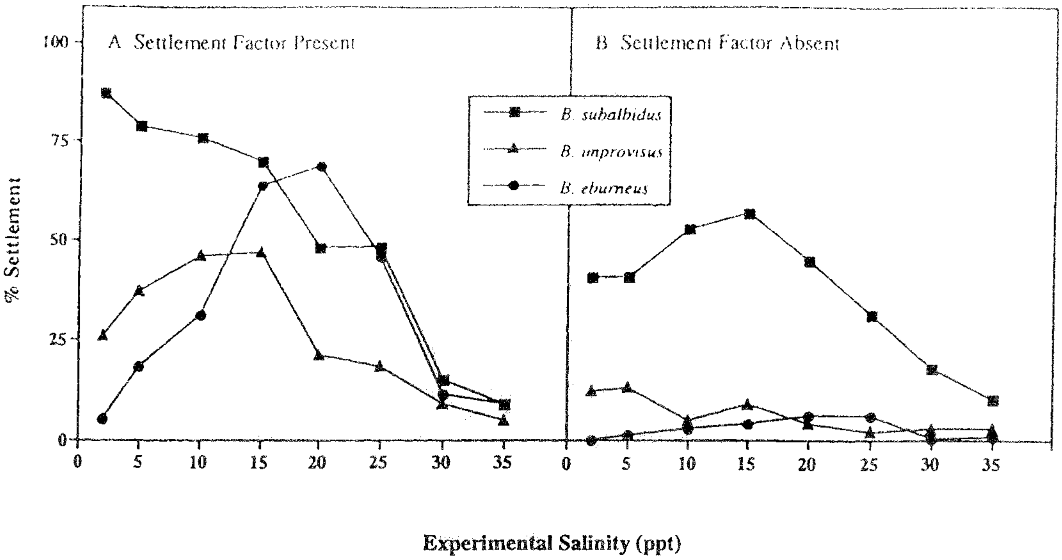

Fig. 4. Comparative perecnt settlement of *Balanus* congeners in the presence (A) and absence (B) of conspecific settlement factors at various test salinites (comparative data extracted from Dineen & Hines, 1992, 1994).

interaction of conspecific chemical and salinity cues (Fig. 4a) than either *B. eburneus* or *B. improvisus*. Although peak settlement of oligohaline *B. subalbidus* occurred at 2 ppt salinity, we unexpectedly found that this congener was stimulated in the presence of conspecific settlement factor to settle over a broad range of salinities up to 20–25 ppt (Fig. 4a), where it does not occur in any abundance in the field. Because of this disparity between settlement and adult distribution, we previously hypothesized that the distribution of adult *B. subalbidus* could be a result of larval retention in oligohaline waters (Dineen & Hines, 1994). In addition, cyprids of *B. subalbidus* were stimulated to settle at relative abundance at mid-salinities in the absence of adult chemical cue, although salinity alone did not stimulate settlement of its congeners (Fig. 4b).

4.2. Postponing metamorphosis

Many invertebrate larvae respond in varying degrees to external cues to settle and metamorphose. In the absence of such a stimulus, competent larvae of many taxa are able to delay metamorphosis (Pechenik, 1990). After postponing metamorphosis, larvae often will settle in a less discriminating manner (Rittschof et al., 1984), possibly because of depleting energy reserves (Lucas et al., 1979), which may jeopardize post-metamorphic growth and/or survival (Pechenik & Cerulli, 1991; Pechenik et al., 1993). For example, we observed a less discriminating pattern of settlement relative to salinity in the presence of conspecific settlement factor when metamorphic delay was artificially imposed upon cyprids of *B. improvisus* (Dineen & Hines, 1992) but not of *B. subalbidus* (Dineen & Hines, 1994). Both untreated and metamorphically delayed cypris cohorts of *B. subalbidus* showed a similar settlement pattern relative to salinity, and lack of discriminating settlement behavior in "delayed" cyprid larvae of this species suggests independence from adult chemical and salinity cues, compared with *B. improvisus*. (Dineen & Hines, 1994).

We expected metamorphically delayed *B. eburneus* cyprids to exhibit a less discriminating settlement response relative to salinity as we observed with *B. improvisus* cyprids, because of the similarity of their larval response to an apparent synergistic interaction of adult extract and salinity (Fig 4a). However, similar to *B. subalbidus*, little difference in settlement pattern relative to salinity was observed between similarly treated cohorts of *B. eburneus* cyprids (Fig. 2). Overall settlement increased in the metamorphically delayed cohort of *B. eburneus* cyprids. In contrast, both *B. improvisus* and *B. subalbidus* showed a decrease in overall settlement when larvae were temporarily prevented from metamorphosing (Dineen & Hines, 1992, 1994). Together, these settlement responses of *B. eburneus* cyprids could suggest that delaying metamorphosis would not be detrimental but rather would be a beneficial strategy for *B. eburneus* larvae. However, since larvae were prevented from metamorphosing by placing them in the cold, it could be argued that *B. eburneus* cyprids are physiologically more capable of coping with the stress of this particular delay. (The northern portion of the geographic range of *B. eburneus* extends to Northeast United States.) Furthermore, post-metamorphic events (i.e. growth rates and or survival), in either larval group, were not assessed and could conceivably differ. A further investigation of the effects of delayed metamorphosis in *B. eburneus*, perhaps employing other delay techniques, would be useful.

4.3. *Settlement in the presence of conspecific and congeneric extract*

When comparing settlement inducement of conspecific and congeneric settlement factor, as expected, *B. eburneus* cyprids were stimulated to settle most abundantly in the presence of conspecific extract (Fig. 3). The species specificity of this response was demonstrated over four decades ago with larvae of *Semibalanus balanoides* [= *Balanus balanoides* (L.)], *B. crenatus* (Brugiere) and *Elminius modestus* (Darwin) (Knight-Jones, 1953) and more recently with larvae of *B. amphitrite amphitrite* (Darwin) (Crisp, 1990), *B. improvisus* (Dineen & Hines, 1992) and *B. subalbidus* (Dineen & Hines, in review). We also demonstrated previously that cyprids of *B. improvisus* settled in greater numbers in the presence of *B. subalbidus* as opposed to *B. eburneus* extract (Dineen & Hines, 1992); and, similarly, settlement response of *B. subalbidus* cyprids was greater in the presence of *B. improvisus* as opposed to *B. eburneus* extract (Dineen & Hines, 1994)

Table 1
Comparative proportional settlement among *Balanus* congeners relative to conspecific and congeneric adult extracts

Species of cyprid	Source of adult extract		
	B. subalbidus	*B. improvisus*	*B. eburneus*
B. subalbidus	1.00	0.56	0.23
B. improvisus	0.68	1.00	0.44
B. eburneus	0.48	0.55	1.00

Proportions were derived by normalizing settlement levels in the presence of conspecific settlement factor for each species (comparative data extracted from Dineen & Hines, 1992, 1994).

(Table 1). Variation in settlement stimulation in the presence of congeneric extract does not correlate with phylogenetic affinities (see Henry & McLaughlin, 1975) of these estuarine congeners (Dineen & Hines, 1992, 1994). In the present study, we demonstrated that cyprids of *B. eburneus* did not distinguish between extract obtained from *B. improvisus* or *B. subalbidus* (Fig. 3), although *B. eburneus* is more closely allied to *B. subalbidus* (Henry & McLaughlin, 1975). Because the cyprid antennule responds with specificity to settlement factor protein, an equivalent settlement response of *B. eburneus* to both congeners could imply a similarity between settlement factor protein derived from *B. improvisus* and *B. subalbidus*. Moreover, this similarity could account for the reciprocal settlement stimulation of *B. improvisus* and *B. subalbidus* in the presence of extract derived from one another (Table 1).

4.4. *Implications of comparative settlement patterns*

Settlement behavior of two of these congeners (*B. eburneus* and *B. improvisus*) could be influential in structuring their estuarine distribution, because their settlement responses to variations in salinity coincide with patterns of their adult salinity distributions in the estuary. In contrast, settlement of the third congener (*B. subalbidus*) probably does not determine adult distribution along the estuarine gradient, because its cyprids respond to a much broader range of laboratory salinities than those where the adults are found in the estuary. For *B. subalbidus*, adult distribution could be caused either by pre-settlement larval behavior resulting in retention of cyprids within a narrow salinity zone, or by differential post-settlement mortality of juveniles at higher salinities.

These studies provide one of the few experimental analyses of interactions among physical and biological factors influencing settlement of larval barnacles. Our experiments indicate that salinity, adult extract and metamorphic postponement all have significant interactive effects upon cyprid settlement. In addition, these experiments allow us to formulate specific hypotheses about the role of settlement in determining adult distribution in the field. However, we recognize the importance of many other variables, e.g. other chemical cues (Raimondi, 1988), surface texture (LeTourneux & Bourget, 1988), and water flow (Mullineaux & Butman, 1991), which are likely to have similar interactive effects (Wethey, 1986) upon barnacle settlement. Our ability to predict settlement behavior in realistic conditions will depend on further analysis of these interactions.

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