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Larval Settlement of the Barnacle, *Balanus improvisus* Darwin (1854) under Different Food Concentration, Substratum, Light Period, Salinity, Cyprid Density and Cyprid Age

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Abstract: The effect of different food concentration, substratum, light periodicity, salinity, cyprid density and cyprid age on the settlement of *Balanus improvisus* were studies. Released larvae were mass reared in 0.6 L glass beakers to the cyprid stage. Then, attachment was monitored once a day. *Chaetoceros calcitrans* was used as food at three cell concentrations (0.5×10^5, 1×10^5 and 2×10^5 cells mL^{-1}). Larval settlement was carried out on two substratum under three light period (12L:12D, 16L:8D and 8L:16D) at seven salinities (7, 12, 15, 18, 25, 28 and 36 ppt). Cyprid density was used from five to 200 larvae per 5 mL of seawater. Cyprids were aged for one to seven days. The maximum and minimum percent settlement was observed at 2×10^5 and 0.5×10^5 cells mL^{-1}, respectively. Cyprids settled in higher numbers on polystyrene compared to glass surface. Percent settlement was higher in 8L:16D than another light periods but, no significant difference in percent settlement between 12L:12D and 16L:8D light periods was seen. Differences in settlement at various salinities were not statistically significant except in 10 ppt which percent settlement was significantly higher. Cyprid density did not have any effect on percent settlement but cyprid age had a significant effect on percent settlement.

Key words: Larval settlement, *Balanus improvisus*, food concentration, light period, salinity, cyprid density

INTRODUCTION

Barnacle larvae are meroplanktonic, staying in the plankton for a restricted period of time and then undergoing settlement and recruitment processes to become sessile adults (Chan and William, 2003). Settlement is the process by which the cypris larva contacts and attaches itself to the substratum and then undergoes metamorphosis to become a sessile individual (Caffey, 1985; Connell, 1985). The cypris larvae are non-feeding and have to depend upon the energy reserves incorporated during the earlier planktotrophic naupliar development (Anil *et al*., 2001).

Larvae of barnacles have been widely used as a model organism for studying the invertebrate larval development, settlement and metamorphosis (Rittschof *et al*., 1992). The choice of substratum is critical for survival of species that cycle between a planktonic larval to a sessile adult condition. The choice of settlement substratum is modulated by complex interactions between environmental variables including biological, chemical and physical cues, light, food availability and the presence of conspecific adults (Barnes, 1970; Crisp, 1974; Pawlik, 1992; Walker, 1995; Clare, 1996; Underwood and Keough, 2001; Faimali *et al*., 2003; Fraschetti *et al*., 2003; Faimali *et al*., 2004).

Laboratory rearing of barnacle larvae under controlled conditions has made it possible to study the influence of various environmental factors such as food concentration, water temperature, light period, salinity, cyprid age and cyprid density, on the larval development and attachment.

A number of investigators have used larvae of *Balanus improvisus* in the laboratory for biofouling research (Dineen and Hines, 1991; Korn, 1991; O'Connor and Richardson, 1994 and 1996; Berntsson *et al*., 2000; Dahlstrom *et al*., 2004; Qvarfordt *et al*., 2006). Investigation comparing the settlement behavior of *B. amphitrite*, *B. improvisus* and *B. eburneus* showed that the settlement behavior of cyprids cannot be generalized (O'Connor and Richardson, 1994), which indicates that the bioassay procedures used for *B. amphitrite* larvae would not be suitable for other species.

B. improvisus is the only barnacle species which inhibits in the South Caspian Sea and information about its settlement is insufficient. The aim of this study was to produce data on larval settlement of *B. improvisus*, in order to standardize a larval bioassay protocol that can be

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used to evaluate candidate antifouling compounds and techniques under laboratory condition in those areas where this species is a dominant species such as Caspian Sea.

MATERIALS AND METHODS

Rearing the syprids: Adult individuals of *B. improvisus* were collected from Noor coast, South Caspian Sea and then transferred to the laboratory in Tarbiat Moalem University. Specimens were placed in an aquarium and kept in constant temperature room (25±1°C) under continuous light. Owing to positive phototaxy of early nauplius stages, hatched nauplii were concentrated to the corner of aquarium using a desk lamp. Then larvae were transferred to 0.6 L glass beakers (1 larvae per 2 mL), using filtered seawater (salinity = 12-13‰, 1 μm filtered and UV-treated) on a daily monoloalgal diet (*Chaetoceros calcitrans*) to cyprid.

Cyprid attachment assay: Newly transformed cyprids (0 day old) were immediately exposed to attachment assay. The assay procedure described by Rittschof *et al*. (1992) was used. Sterile polystyrene Petri dishes (depth = 10, diameter = 60 mm) were used as the substratum for settlement. Under the dissection microscope, an exact number of cyprids (= 25) were introduced together with 5 mL of Filtered Sea Water (FSW) and incubated for 24 h at 25±1°C. Six replicate were used for each treatment. All experiments were repeated twice using different batches of cyprids, since the results of both repeats showed statistically the same trend only first repeat data are presented.

The effect of algal food concentration on the duration of larval development, cyprid yield and settlement was investigated as following procedure. Nauplii were reared to cyprids in batches of 250 larvae, using the procedure described earlier. The concentration of the food (*C. calcitrans*) used (for 1 larva per 2 mL) were 0.5×10^5, 1×10^5, 2×10^5 and 3×10^5 cells mL^{-1}. Three replicate cultures were used for each feeding regime. The algal concentration was checked regularly (once every 12 h) using a haemocytometer and attuned until cyprid appearance. Survival rate (cyprid yield) was accounted at the end of each experiment. Sterile polystyrene (depth = 10 mm; diameter = 60 mL) and borosilicate glass (depth = 10 mm; diameter = 60 mL. Petri dishes were used as the test substrata to study the cyprid preference for hydrophobic and hydrophilic surfaces, respectively. To determine the effect of light on settlement, cyprids were incubated under three light periods includes: 12L:12D, 16L:8D and 8L:16D. Light was provided using 40 W fluorescent lamps (2500-3000 Lx). The effect of seawater salinity on settlement was determined by allowing the cyprids to settle at the various salinities contain: 7, 12, 15, 18, 25, 28 and 36 ppt. Seawater was either diluted with double distilled water or evaporated to obtain the required salinity. The effect of cyprid density on settlement was investigated by incubating 5-200 cyprids per 5 mL of seawater. Newly transformed cyprids were aged for one to seven days, by placing them in an incubator at 6°C under darkness. Every day, a few cyprids were warmed to room temperature and used for the settlement assay.

Statistical analysis: The normality and homogeneity of variance were checked with Shapiro-Wilk's and Cochran's test, respectively (Zar, 1999). The effects of food concentration, light period, salinity, Cyprid age and cyprid density on larval attachment were evaluated using one-way ANOVA and then means were compared with Tukey's test.

RESULTS

The majority of nauplii II larvae which fed on *Chaetoceros calcitrans* completed the life cycle to cypris stage within 6-7 days. Larvae fed on *Cha. calcitrans* at the concentration of 1×10^5, 2×10^5 and 3×10^5 cells mL^{-1} show shorter time interval (6 days) and higher cyprid yield than those fed at 0.5×10^5 cells mL^{-1} (7 days). An on-way ANOVA indicated food concentration used for larval development influenced the attachment significantly. The maximum and minimum percent settlement was observed at 2×10^5 and 0.5×10^5 cells mL^{-1}, respectively (Fig. 1).

Regardless of cyprid age, cyprids settled in higher numbers on polystyrene (67±3%) compared to glass (45±3%) surface.

Percent settlement was higher in 8L:16D (75±4%) than another light periods but, no significant difference in percent settlement between 12L:12D and 16L:8D light periods was seen (Fig. 2).

Maximum and minimum percent settlement was observed at 12 and 36 ppt, respectively. But, Differences in settlement at other salinities were not statistically significant (Fig. 3). Also, cyprid density (from 5-200 larvae per mL of FSW) did not have any effect on percent settlement ($F_{6,35} = 4.5$; $p = 0.08$).

There were significant differences in the percent settlement between age groups include 0 to 7 days.

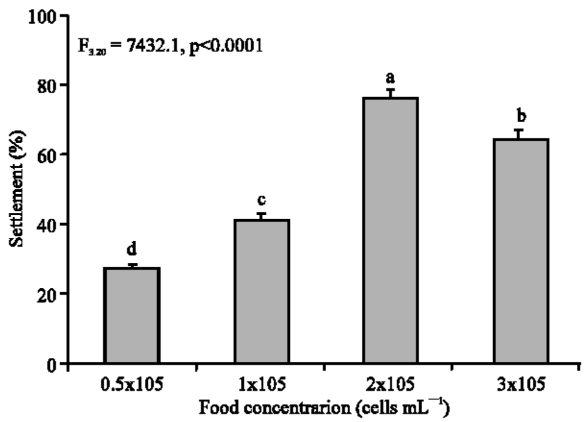

Fig. 1: Effect of food (*Chaetoceros calcitrans*) concentration used during naupliar development on the settlement of the cyprids. Each data point represents the mean (±SD) of six replicates. Data that are significantly different at α = 0.05 in Tukey's test are indicated by different letter above the bar

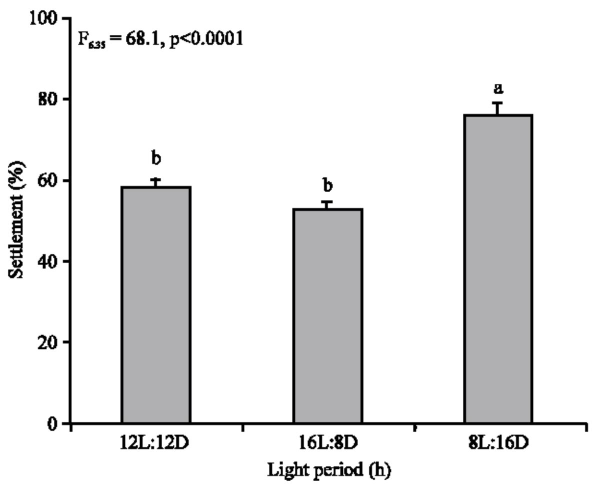

Fig. 2: Effect of light periods on the settlement of the cyprids. Each data point represents the mean (±SD) of six replicates. Data that are significantly different at α = 0.05 in Tukey's test are indicated by different letter above the bar

Percent settlement from 0 day old due to 4 day old was increasingly whereas, after 4 day old percent settlement tended to reduction. Maximum settlement was recorded in four day old cyprids and minimum was documented in 0 day old cyprids (Fig. 4).

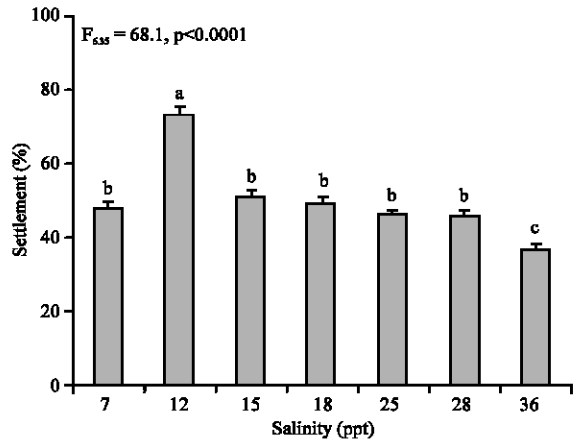

Fig. 3: Effect of salinity on the settlement of the cyprids. Each data point represents the mean (±SD) of six replicates. Data that are significantly different at α = 0.05 in Tukey's test are indicated by different letter above the bar

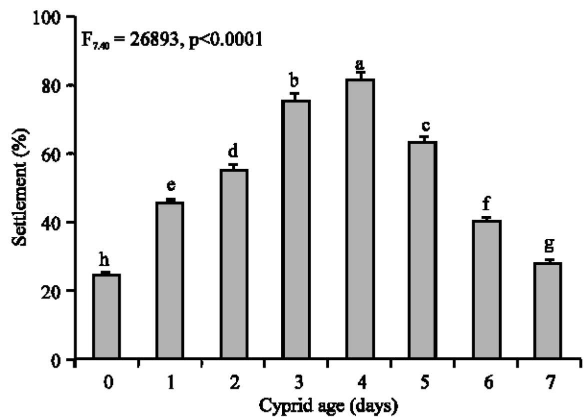

Fig. 4: Effect of cyprid age on the settlement of the cyprids. Each data point represents the mean (±SD) of six replicates. Data that are significantly different at α = 0.05 in Tukey's test are indicated by different letter above the bar

DISCUSSION

Once larvae are transported to a particular location and begin to encounter suitable substrata, their settlement behavior can become important and invertebrate larvae can respond to a large number of cues, including physical features such as light and salinity (Crisp and Ritz, 1973; Dineen and Hines, 1994); physical properties of the substratum such as substratum topography (Crisp and Barnes, 1954; Williams, 1965; Crisp and Ryland, 1960;

Wethey, 1986; Wright and Boxshall, 1999) and small-scale hydrodynamic characteristics (Williams, 1965; Mullineaux and Butman, 1991). Larvae can also respond to biological cues such as microbial films (Crisp and Ryland, 1960; Keough and Raimondi, 1995) and conspecific individuals (Knight-Jones and Stephenson, 1950; Crisp and Meadows, 1962; Wethey, 1984; Bayliss, 1993; Minchinton, 1997; Wright and Boxshall, 1999).

In the present study larvel settlement of *Balanus improvisus* was observed under different physical and biological features and therefore, optimal condition was recorded for this species in the laboratory. The amount of cyprid energy reserves depends on the naupliar feeding history, specifically on algal food quantity and quality. Studies relating barnacle larval development to algal food availability is usually restricted to food quantity (West and Costlow, 1987; Anil and Kurian, 1996; Qiu and Qian, 1997; Hentschel and Emlet, 2000) while factors related to food quality have been insufficiently addressed. Our results show that quantity of algal food provided to naupliar larvae had a clear impact on the duration of larval development as well as on cyprid yield (survival). Thisfinding was supported by West and Costlow (1987) observation in the case of *B. eburneus*. Each stage of larval development requires a minimum level of energy to metamorphose into the next stage (West and Costlow, 1987). However, algal cell above the optimum concentration may hinder the filtration process. These can explain why *B. improvisus* larvae performed poorly settlement at food concentration $<1\times10^5$ and $>2\times10^5$ cells mL^{-1}.

O'Connor and Richardson (1994) reported that cyprids of *B. improvisus* attach to a lesser extent to glass vials than to polystyrene dishes. These results are corresponding with our findings.

Light is considered to be an important physical indication to which barnacle larvae can respond (Crisp and Ritz, 1973). A few observations made in the field at different times and with different species suggest that light plays an important role in determining settlement of cyprids. The number of settling cyprids of *B. eburneus* (McDougall, 1943 in De Wolf, 1973), *B. improvisus* (Weiss, 1947 in De Wolf, 1973) and *Chthamalus anisopoma* (Raimondi, 1990) was higher during the day than during the night. The same pattern was observed with *Chthamalus* species in a preliminary study done in the SW coast of Portugal (Cruz, 1999). In contrast, De Wolf (1973) also cited a few barnacle studies where this pattern was not evident. Wethey (1984), when observing variability in tidal settlement of *Semibalanus balanoides*, did not suggest any influence of the night/day cycle. The number of settling cyprids of *B. reticulatus* during darkness was higher than during the light (Thiyagarajan *et al*., 2002). Results of our study were corroborated investigation of Thiyagarajan *et al.* (2002). However, observations made during these studies were at restricted locations and over a short duration.

Post settlement osmotic/ionic stress of juvenile barnacles could potentially be a factor in influencing recruitment (Thiagarajan *et al*., 2002). In this study salinity has not much effect on settlement of *B. improvisus* cyprid. Since this species can be characterized as a strong osmooregula-because of its adaptation rapidly to the new salinities when exposed to different salinities - (Nasrolahi et al., 2006, lower change in its settlement at moderately wide range of salinities is to expected. This results is similar to results of Thiagarajan *et al.* (2002) on *B. reticulates*. Cyprid density is not very significant in the settlement of *B. improvisus* larvae because varying the number of cyprids in the range of 5-200 per 5 mL of seawater does not affect the percent settlement. The same result was observed by Thiyagarajan *et al.*(2002) but cyprid density has clear impact in the settlement of *B. aniphitrite* larvae (Clare *et al*., 1994).

Besides energetic factors, attachment of cyprids is significantly controlled by a temporal component, i.e., the age of cyprids. Newly transformed cyprids respond to settlement factor (SF) (Rittschof *et al*., 1984), but may postpone attachment in the absence of a suitable substratum (reviewed by Pechenik, 1999). In contrast, old cyprids attach on otherwise less acceptable substrata to assure survival (Crisp, 1988). Due to the clear correlation of the physiological condition of cyprids and metamorphic success, recruitment of barnacles is determined at least to some extent by endogenous factors (Raimondi, 1990; Jarrett and Pechenik, 1997; Miron *et al*., 1999, 2000). 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. The same trend was seen in this study. The investigation of Anil *et al.* (2001) showed 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.

Generally, in according to results of this study, cyprid larvae of *B. improvisus* can be one of the good candidates for antifouling assay-especially in regions such as Caspian Sea which only this species is presented-as a consequence of easy culture in the laboratory.

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