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Larval Settlement and Spat Growth of the Tropical Oyster *Crassostrea belcheri* (Sowerby 1871) in Response to Substrate Preparations

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Abstract

Substrate preparation is a key factor for the settlement and metamorphosis of oyster larvae. In the present study, the effects of substrate preparations on larval settlement and growth of spat of the tropical oyster, *Crassostrea belcheri* were evaluated in two trials in a semi-closed recirculation system. In each trial, substrates were prepared by three different methods, including immersion in seawater for 2 hr, conditioning with a biofilm, and presoaking in adult tissue extracts. The number of larvae that settled on substrate presoaked in adult tissue extracts or which had a biofilm were significantly higher ($P<0.05$) than the number that settled on substrates immersed in seawater for 2 hr in both trials. Mean settlement rates were 30.53±2.91, 38.52±2.81 and 40.86±3.65% in trial 1, and 19.02±2.49, 28.66±3.13 and 34.13±2.91% in trial 2 for substrates prepared by immersion in seawater for 2 hr, with a biofilm, and presoaked in tissue extracts, respectively. Differences in the increments of shell width and length among oyster spat nursed over 4 weeks were non-significant ($P>0.05$) among the treatments.

Introduction

Tropical oyster, *Crassostrea belcheri* (Sowerby 1871), is one of the most commercially important bivalves in Thailand. The majority of spat for grow-out farms are collected from natural sources, but the amount of oyster seed produced from those sources is limited and insufficient. Oyster seed production from hatcheries is being developed and is the subject of great interest in Thailand. Difficulties in bivalve seed production under hatchery conditions are mainly associated with settlement and metamorphosis (Helm et al. 2004). Attachment to substrate by bivalves is not a completely random event and suitable substrate must be provided for larvae to metamorphose. A wide variety of environmental factors, particularly the nature of the substratum and the presence of some dissolved compounds, both biotic and chemical, have been found to be capable of inducing settlement and metamorphosis of larva of different species of marine bivalves (García-Lavandeira et al. 2005). The preferred settlement surfaces are rough-textured substrates, covered with a biofilm (Maki and Mitchell 1985; Weiner et al. 1989; Tamburri et al. 1992; Pearce and Bourget 1996;

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Taylor et al. 1998; Zhao et al. 2003; Peteiro et al. 2007; Su et al. 2007), conspecifics (Jensen et al. 1990; Zimmer-Faust and Tamburri 1994; Devakie and Ali 2002; Khandeparker et al. 2003; Su et al. 2007), and some particular chemical substances (Anderson and Underwood 1994; Anderson 1996; Zhao et al. 2003). However, a suitable substrate and the natural cues for settlement and growth appear to be species specific (Gosling 2003). The objective of the experiment described in this study was to determine ways of maximizing larval settlement and growth of spat with different substrate preparations. The best substrate preparation technique found in this study can be used to achieve higher settlement rates in *C. belcheri* larvae under hatchery conditions.

Materials and Methods

Experimental system

A semi-closed recirculation system was designed to determine the effect of substrate preparations on larval settlement and spat growth of the tropical oyster, *C. belcheri*. The system consisted of a submersible pump, a 1,500 L fiber glass tank (dimensions 1.10 x 2.20 x 0.62 m) used to hold setting units, a 105 L fiber glass tank (dimensions 50 x 70 x 30 cm) used for water storage, and 9 sets of 29 L fiber glass rings (diameter 35 cm x length 30 cm) used as setting units. Screen with a mesh size of 180 µm was fixed by a fiber glass clamp to the bottom of each fiber glass ring.

During the experiment, the water from the water storage tank was pumped into the large tank where the setting units were placed. The water was injected so that it down-welled into each setting unit and then drained though the overflow pipe before being returned to the storage tank. The rate of water flow in each setting unit was adjusted by the valve on the inflow pipe to 0.5 $L \cdot min^{-1}$. Water salinity was maintained at 30 ppt over the study period. Water quality parameters were as follows: dissolved oxygen > 6.76 $mg \cdot L^{-1}$, total ammonia nitrogen < 0.16 $mg \cdot L^{-1}$, water temperature 25.5-27.2 ^{o}C and pH $\geq$ 7.84.

Experimental oyster larvae

Mature *C. belcheri* oysters were collected from Kantang district, Trang province. The collected oysters were cleaned and acclimated in sand-filtered seawater (30 ppt) in the hatchery for 2 days. Broodstock were conditioned in 1,000 L fiberglass tanks with an algal mixture containing 6% *Chaetoceros calcitrans* and *Tetraselmis suecica* per mg oyster (dry algal weight·dry meat $weight^{-1}$). Two weeks later, gametes were obtained from sacrificed oysters, and the eggs were fertilized using 1 male: 4 females. The fertilized eggs were stocked for further development in 500 L culture tanks at a density of 15 $embryos \cdot mL^{-1}$ in filtered (1 µm) and UV-treated seawater. The embryos developed to D larvae within 2 days, and the stocking density was reduced to 5 $larvae \cdot ml^{-1}$. The D larvae were fed daily with *Isochrysis galbana* at a density of 20,000 $cells \cdot mL^{-1}$. Seawater renewal and tank cleaning was carried out on alternate days, along with the addition of antibiotics (Helm et al. 2004). At each draining, larvae were sieved to grade the size. When the size of larvae was greater than 100

µm, a mixed diet of *I. galbana* and *C. calcitrans* was fed daily and the algal density was increased to 50,000 cells·ml^{-1} (Charlermwath, 2001). The pediveliger stage (competent larvae with the presence of an eyespot) was attained after 18 days of culture. Ready to set pediveligers were graded with a 250 µm mesh sized sieve. Pediveligers which retained on the 250 µm nitex sieve were then transferred to a 10 L plastic bucket and counted prior to use in experiments. Two trials were carried out with a completely randomized design (CRD). Three different substrate preparations were used in each trial where each treatment was carried out in triplicate. Each setting unit was stocked with 40,000 larvae or 2,000 larvae·L^{-1}

Substrate preparation

The particulate material used as substrate for oyster setting was made from clean oyster shells, which had been sun dried and broken into particles using a stone mortar and pestle. The particles were graded so that only those that passed through a 500 µm screen but which retained on a 250 µm screen were used as substrate for oyster setting in the experiments. Three different methods of substrate preparation were used in this experiment, including immersion in seawater for a duration of 2 hr, conditioning with biofilm, and with adult tissue extracts.

Conditioning substrate in seawater

New substrate was prepared as described above and cleaned with freshwater before being spread over the screen in the setting unit, and conditioned in the system for 2 hr prior to release of the oyster larvae. A previous experiment showed that the highest settlement rate of oyster larvae (*C. belcheri*) occurred on substrates conditioned for 2 hr under hatchery conditions (Tanyaros, 2011).

Conditioning substrate with a biofilm

Substrate was acid washed with 10% HCl and pressure cleaned with freshwater. Then it was placed in a 10 L aerated culture of the diatom *C. calcitrans* for 72 hr to allow development of an epifloral biofilm (Taylor et al. 1998). Seventy-two hours had previously been shown to be the time required for biofilms to become most attractive to oyster (Ostreidae) larvae (Weiner et al. 1989).

Conditioning substrate in adult tissue extracts

Conditioning of substrates with adult tissue extracts of the same species has resulted in higher settlement rates for another species of tropical oyster (*Crassostrea iredalei*) (Devakie and Ali, 2002). In this study, the tissue extract of adult *C. belcheri* was prepared following the method of Devakie and Ali (2002) and Khandeparker et al. (2003). Ten adult oysters were shucked, and the meat blended and made up to 1 L in a standing measuring cylinder, and then left overnight in a low heat incubator at 4 ^{o}C. The supernatant (about 100 mL of the top layer) was poured off and made up to 0.5 L and the substrate immersed in that solution for 24 hr. The substrates were then air dried for another 24 hr period prior to use for larval settlement.

Settlement and growth assays

Two days after being allowed to set, post-larvae on the substrate in each setting unit was sampled by resuspending the substrate and then immediately placing a petri dish plate (diameter 3.10 cm) on the bottom of the setting unit. After all the substrate had settled, the petri dish plate was removed and the number of post-larvae on the substrate was counted using a binocular stereomicroscope. The total number of larvae set on the substrates was calculated by multiplying the number of post-larvae per unit area (1 cm^{-2}) of the petri dish by the total area of the setting for unit (962.54 cm^{-2}). The numbers of attached spat were expressed as a percentage of settlement (100 x total number of larvae settled/total number of larvae). After assaying the settlement, the experimental system and setting units containing substrate and oyster spat were cleaned with sea water. The spat were then allowed to grow for 4 weeks to determine the effect of various substrate preparations on growth. During this part of the study, the direction of water flow in the down-welling was switched to up-welling, and the flow rate in each setting unit was increased to 7.5 $L \cdot min^{-1}$. Total water change was carried out on alternate days and microalgae mixture consisting of *C. calcitrans* and *T. suecica* was added into the water storage tank in the ratio of 50:50 at a rate of 25,000 $cells \cdot mL^{-1}$, respectively twice a day (morning and evening) Twenty samples of oyster spat from each replicate were taken weekly to measure the shell width (dorso-ventral measurement) and length (antero-posterior measurement) using a binocular stereomicroscope. The ocular scale on the stereomicroscope was calibrated using a micrometer prior to use for measurement.

Statistical analysis

Data from the experiments were analyzed statistically to test for differences among conditioning methods using an analysis of variance (one-way ANOVA) from the Analytical Software SPSS 11.5 for Windows. If significant effects were presented, a further analysis using the Duncan's multiple range test (DMRT) was used to determine the pairwise comparison of their means.

Results

Larvae settlement

Settlement rates obtained under different methods of substrate preparation are shown in Fig. 1. Mean percentage settlement differed significantly among treatments ($P<0.05$) (Table 1), with the highest occurring on substrate presoaked in tissue extracts and the lowest on substrate immersed in seawater for 2 hr. However, no significant difference ($P>0.05$) in settlement rates was found between substrates with a biofilm and those presoaked in tissue extracts. In the two experimental trials, mean percentage set were 30.53±2.91, 38.52±2.81 and 40.86±3.65 in trial 1, while mean percentage set were 19.02±2.49, 28.66±3.13 and 34.13±2.91 in trial 2, on substrates prepared by immersion in seawater for 2 hr, with a biofilm, and presoaked in tissue extracts, respectively.

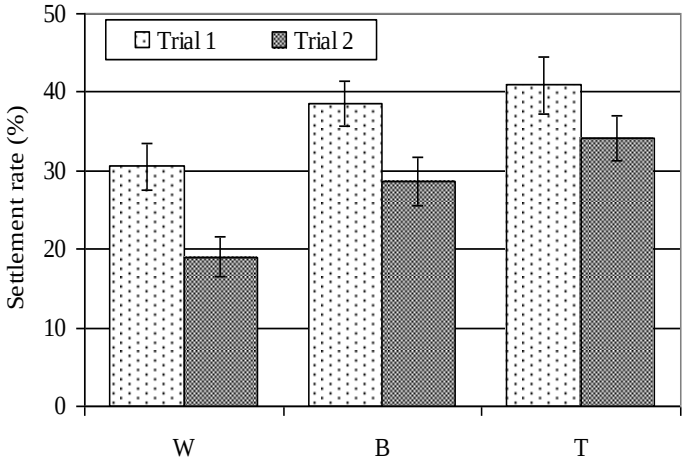

Fig. 1. Percentage of *C. belcheri* larvae which set on substrates prepared by different methods (W = immersion in seawater for 2 hr, B = with a biofilm, T = presoaked in tissue extracts).

Table 1. The one-way ANOVA analysis of the settlement of *C. belcheri* larvae on substrates prepared by different methods (W = immersion in seawater for 2 hr, B = with a biofilm, T = presoaked in tissue extracts).

		SS	***df***	**MS**	***F***	***P***
Trial 1	Substrate preparation (W, B, T)	176.147	2	88.073	8.898	0.016
	Error	59.386	6	9.898		
	Total	235.533	8			
Trial 2	Substrate preparation (W, B, T)	350.120	2	175.060	21.372	0.002
	Error	49.147	6	8.191		
	Total	399.267	8			

Spat growth

After setting, hatchery-reared spat oysters were nursed in a semi-closed recirculation system for 4 weeks. The initial mean values of shell width and length were 0.34±0.001 µm and 0.32±0.001 mm, respectively. After 4 weeks of nursing, the mean shell width increased to 3.46±0.25, 3.60±0.16 and 3.53±0.28 mm in trial 1, and 4.35±0.19, 4.15±0.27 and 4.13±0.11 in trial 2, for substrates prepared by immersion in seawater for 2 hr, with a biofilm, and presoaked in tissue extracts, respectively (Fig. 2). The mean shell length increased to 3.09±0.21, 3.26±0.14 and 3.25±0.25 mm in trial 1, and 3.87±0.29, 3.64±0.17 and 3.64±0.03 in trial 2, for substrates prepared by immersion in seawater for 2 hr, with a biofilm, and presoaked in tissue extracts (Fig. 3). No significant difference (p>0.05) was found in the daily increment of mean shell width and length among the treatments in both trials (Fig. 4 and 5).

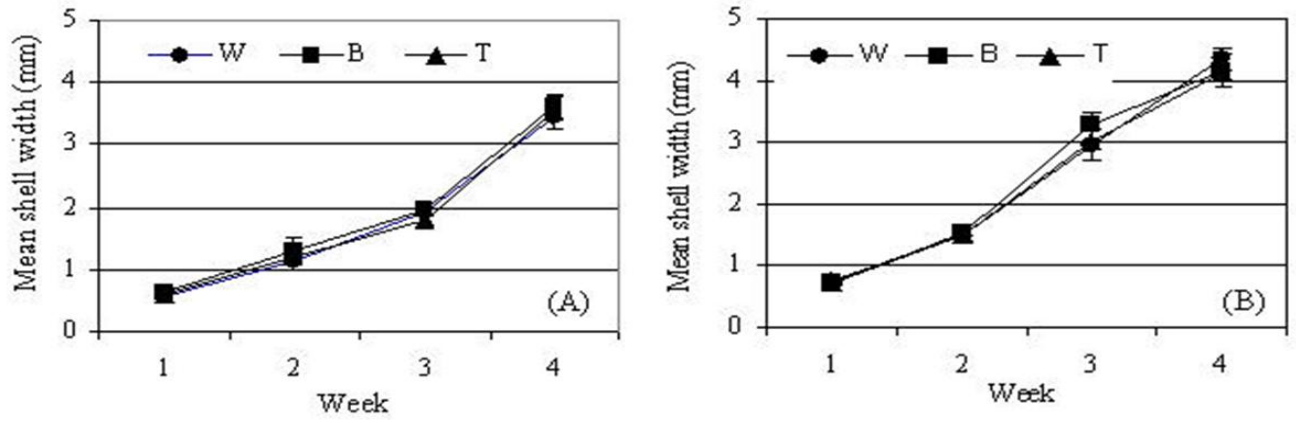

Fig. 2. Mean (±SD) shell width of hatchery-reared spats on substrates prepared by different methods (A = Trial 1, B = Trial 2; W = immersed in seawater for 2 hr, B = with a biofilm, T = presoaked in tissue extracts).

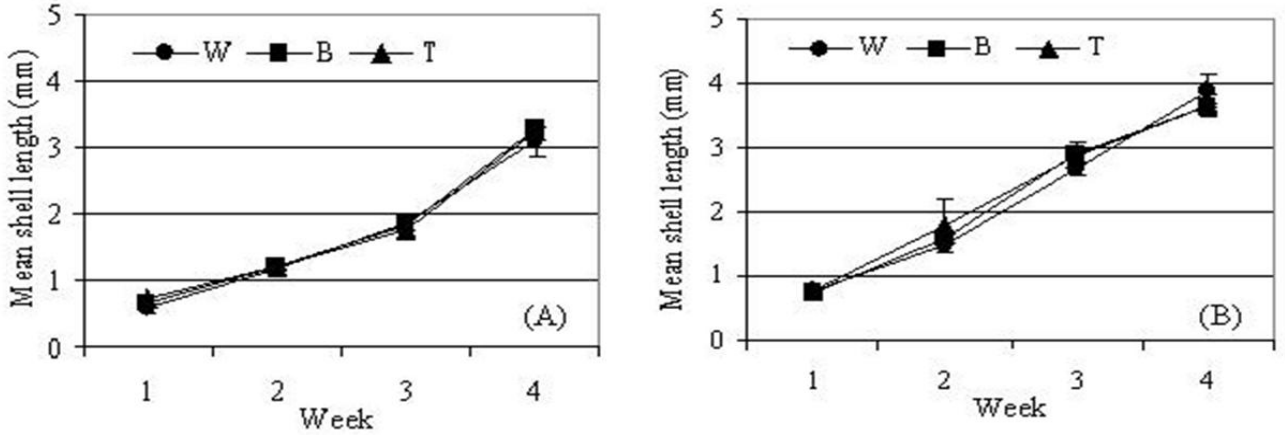

Fig. 3. Mean (±SD) shell length of hatchery-reared spats on substrates prepared by different methods (A = Trial 1, B = Trial 2; W = immersed in seawater for 2 hr, B = with a biofilm, T = presoaked in tissue extracts).

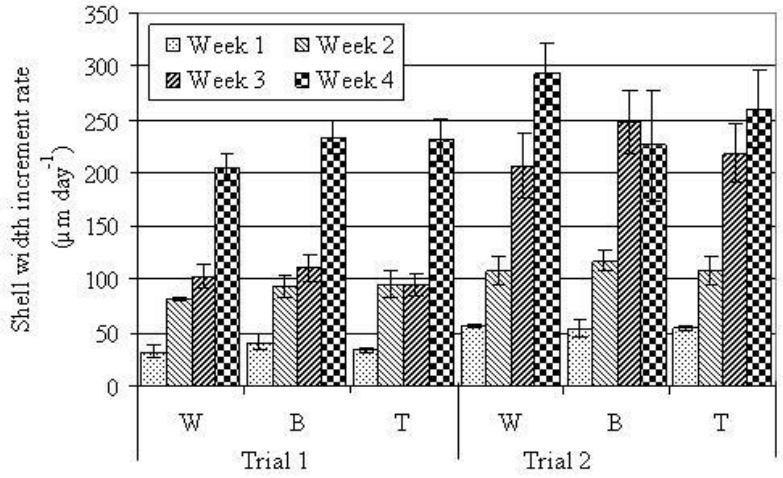

Fig. 4. Mean (±SD) daily shell width increment of hatchery-reared spat substrates prepared by different methods (W = immersed in seawater for 2 hr, B = with a biofilm, T = presoaked in tissue extracts).

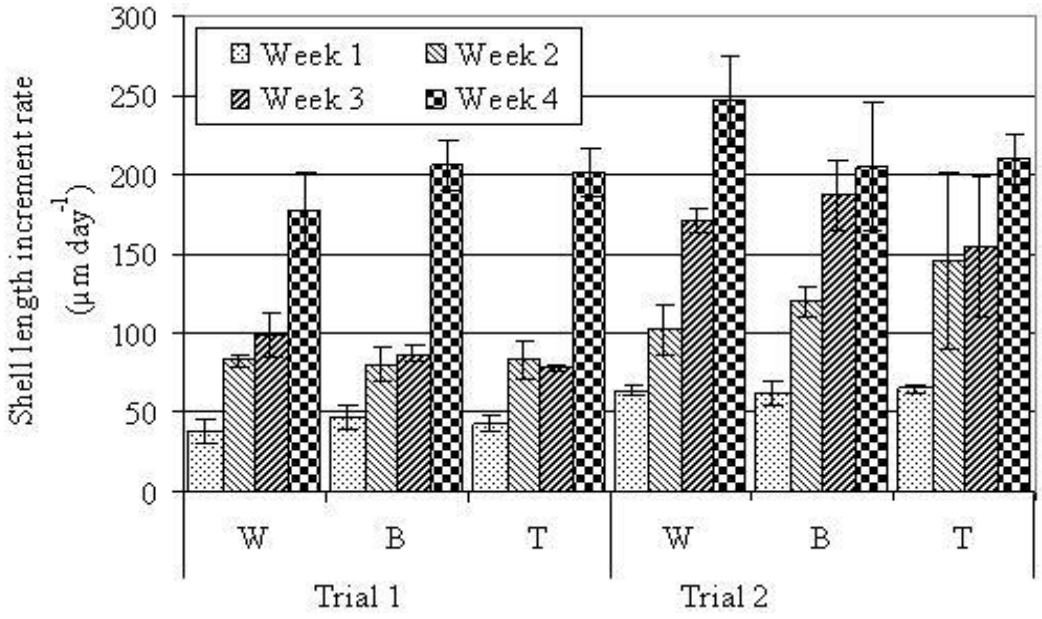

Fig. 5. Mean (±SD) daily shell length increment of hatchery-reared spat on substrates prepared by different methods (W = immersed in seawater for 2 hr, B = with a biofilm, T = presoaked in tissue extracts).

Discussion

The larvae of many marine bivalves preferentially settle and metamorphose in habitats well suited to their subsequent adult life. For many years, researchers have been continuously trying to develop and improve methods of producing bivalve spat. Difficulties with bivalve seed production in hatcheries are mainly associated with settlement and metamorphosis. A number of physical, chemical and behavioural factors have been shown to influence larval settlement in bivalves (Weiner et al. 1989; Fitt et al. 1990; O'Foighil et al. 1990; Tritar et al. 1992; Turner et al. 1994; Zimmer-Faust and Tamburri, 1994; Baker, 1997). In this experiment, higher setting rates for *C. belcheri* larvae on substrate presoaked in tissue extracts from *C. belcheri* were found compared to setting rates on substrate immersed in seawater for 2 hr. Larval settlement of several oyster species has been shown to be induced by conspecific chemical cues in laboratory experiments. For examples, Bayne (1969) reported that leachate from molluscan tissues promoted the settlement of *Ostrea edulis* (Linnaeus) larvae. In a related experiment on another oyster species (*C. iredalei*), Devakie and Ali (2002) showed that substrates coated with tissue extracts of the same species attracted more larvae than those with tissue extracts of other species. Su et al. (2007) showed that the number of setting *Pinctada martensii* larvae was more on plastic sheets coated with adult tissue extracts than on untreated substrates.

Many studies have shown that biofilms play a very important role for triggering larval settlement in many different marine bivalves (Weiner et al. 1989; Tamburri et al. 1992; Pearce and Bourget, 1996; Zhao et al. 2003; Peteiro et al. 2007; Su et al. 2007). In this study, the settlement rate

of *C. belcheri* larvae on substrate with a biofilm was found to be lower than on substrate presoaked in tissue extracts, but the statistical analysis indicated there was no significant difference, which suggested that a biofilm could be equally effective as a stimulate of *C. belcheri* larval settlement. Research on biofilm layers using electron microscopy has shown them to be composed of bacteria, microalgae and detritus (Harvey et al. 1995). O'Foighil et al. (1990) suggested that as well as providing a settlement cue, the biofilm may have provided nutrition to metamorphosing larvae by 'deposit feeding'. In laboratory experiments, Taylor et al. (1998) showed that PVC slats with a biofilm collected more silver-lip pearl oyster (*P. maxima*) larvae than clean PVC slats. Zhao et al. (2003) also reported that natural biofilms induced larval settlement of the silver- or goldlip pearl oyster, *P. maxima*, suggesting that the presence of a biofilm layer on a substrate may provide important chemical cues for larval settlement.

Substrate preparation by different methods in the present study was shown not to influence growth of oysters in the post-setting stage. O'Foighil et al. (1990) stated that bacteria and epifloral films on conditioned substrates were less important than suspended microalgae for early juvenile growth. They found that the Japanese scallop, *Patinopecten yessoensis*, preferentially settled on collector material conditioned with an epifloral film of diatoms, but there was no evidence that the presence of an epifloral film enhanced subsequent spat growth. Therefore, it is expected that algae added into the system as food would have had a more significant effect on the growth of oyster spat in the present study.

Conclusion

In our study, the substrate presoaked in adult tissue extracts and biofilm showed a higher settlement rate of the tropical oyster *C. belcheri* larvae than the substrate immersed in seawater for 2 hr. The differences of substrate preparations were ineffective on growth of oyster spat.

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