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Larvae of the Commercial Tropical Oyster *Crassostrea belcheri* (Sowerby) are induced to settle by Pheromones from the Adults

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ABSTRACT: Pediveliger larvae of the commercial tropical oyster *Crassostrea belcheri* were allowed to settle under different conditions. Two types of seawater were used for the experiments: aged seawater (1 month) and aged seawater conditioned by the presence of adult oysters for 24 hours. Two groups of five different substrates were used, each group soaked in one of the two water types. More larvae settled in conditioned aged seawater than in aged unconditioned seawater and on soaked substrates contrary to unsoaked substrates. The larvae preferred substrates of their own species for settling rather than from other species within the same genus or from a different genus. Settling frequency was increased by the presence of living oysters together with soaked spatfall collectors in commercial aquaculture. The fact that larvae are induced to settle by pheromones released from the adults is an important mechanism that favours the gregariousness of the oysters in nature. Settling behaviour of oyster larvae was observed and the behaviour of the three main stages was described: free swimming searching stage, crawling stage and cementing stage.

KEY WORDS: tropical oyster, larvae settling, pheromones, *Crassostrea belcheri.*

INTRODUCTION

Swimming larvae of many benthic marine bivalves remain in the plankton for a period of time before undergoing metamorphosis to the juvenile stage. This usually occurs in conjunction with settlement. The settlement response in numerous bivalve larvae is regulated by intrinsic and extrinsic factors, including heredity, age, and the nutritional history of the larvae as well as the physical and chemical characteristics of available substrates (Hadfield, 1984). Cole and Knight-Jones (1949) discovered "gregarious setting" in larvae of the European oyster *Ostrea edulis* Linnaeus. The presence of postmetamorphic oysters stimulated the setting of larvae near the site and suggested that a waterborne substance released by the small oysters stimulated the pediveliger larvae to set. Moreover, in a subsequent paper, Knight-Jones

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(1952) showed by statistical analysis that more larvae settled on shells in well stocked oyster grounds than in grounds where the adults were less common or absent. Crisp (1967) demonstrated that shells become very much less favourable for the settlement of larvae of their own species when the periostracum of the outer surface shell and the conchiolin matrix of the inner shell surface of *Crassostrea virginica* (Gmelin) were removed by means of sodium hypochlorite. Extracts prepared by keeping the soft parts of the bodies of *C. virginica* in sea water were capable of making shells somewhat more favorable for settlement. According to Crisp (1967) the larvae may settle gregariously because they recognize both the insoluble organic layer of the shell and solubles emanating from living oysters, but the degree of specificity is unknown.

Some researchers expressed the opinion that the gregarious settling is due to direct contact of larvae with compounds adhering to the settling surface, because small larvae were unable to sense a concentration gradient and because of their slow swimming speed they could not effectively approach the source (Crisp, 1965, 1974, Bayne, 1969). However, later research on the American oyster, *C. virginica,* has indicated action by waterborne pheromones (Hidu, 1969, Keck *et al*., 1970, Veitch and Hidu, 1971). Spat enclosed in bags of plankton mesh too small for the passage of larvae stimulated setting on cultch (spatfall collectors) outside the bags. Addition of extrapallial fluid (EPF) or sea water which had contained adults oysters showed significantly increased settling rates within one or two hours. Of course, there is the possibility that the administered solubles became adhered to the cultch surfaces and the larvae responded to surface contact after all, as suggested by Crisp (1974). Hidu *et al.* (1978) showed that there are significant differences in the response to waterborne pheromones in the gregarious settling of European and American oyster larvae, respectively, to extrapallial fluid (EPF) prior to and during exposure to cultch shells when compared with the control. They also found that the response was very rapid. One or two hours of exposure to such pheromones resulted in more than a doubling of the settling in the experimental cultures. The settling behavior was activated within 10 minutes of the application of EPF.

Pediveliger larvae will search and settle as fast as they can on the preferred substrates and grow through to metamorphosis. However, they can prolong their larval life before settlement if they do not find a suitable substrate. Hidu (1969) found that pediveliger larvae on average settled 7 hours later on substrates without attached spat. Moreover, Hidu *et al*. (1978) showed that cross reactivity of pheromones between *Crassostrea* and *Ostrea* suggested that pheromones released from adults may modify recruitment rates in other species. They also concluded that the interspecific settling response might

be the biological basis for the establishment of many marine benthic communities.

To date nothing has been reported on waterborne pheromones from adult oysters inducing settlement of the larvae of the commercial tropical oyster *C. belcheri.* The aim of this study was to test the settling response of *C. belcheri* larvae in relation to water, soaking conditions and substrate types, to test the effect of oyster pheromones (soluble compounds) and substrate types (insoluble compounds) on the settlement of the larvae.

MATERIALS AND METHODS

Rearing larvae

Larvae of *C. belcheri* were obtained using the standard culture technique described by Wong *et al*. (1989). Eyed larvae were cultured using 1 μm filtered seawater (18 ppt salinity).The culture medium was changed on alternate days. A diet of *Isochrysis galbana* (Parke) (Tahitian strain) and *Chaetoceros simplex* var. *calcitrans* (Paulsen) was provided daily. Metamorphic competence was indicated by the presence of well developed eyes and a predilection to metamorphose on the surfaces of the holding tanks or on cultch provided. Competence to settle in eyed larvae (approximately 14 days old) in the hatchery of *C. belcheri*), was indicated by changing behaviour from swimming to crawling with extension of the foot at the bottom surface in order to find a favorable place for settlement and metamorphosis. The average competent size of larvae of *C. belcheri*, was 278.9 μm (SD = 24.6, n = 105) pediveliger shell length. They were collected in a 253 μm Nitex sieve and used in all experiments.

Seawater types

Water quality is crucial in experiments of this type. If pheromones are present, they are expected to be unstable and short-lived. They would otherwise create confusion in nature. Therefore, we used aged seawater stored for one month to avoid contamination by natural pheromones in the experiments.

Two kinds of water types were tested:
Type A: aged seawater that was conditioned by the presence of living adult oysters 24 hours prior to the experiment. This water was oxygenated by air-bubbling during the conditioning, to keep the oysters alive.
Type B: aged seawater without any additional treatment.

Substrate types

Oyster shells and marble chips were used as settling substrates. The larvae were offered shell fragments of their own species (*C. belcheri,* Cbe*)*, of a different species of the same genus (*C. bilineata*, Cbi), and of a species belonging to a different genus (*Saccostrea cucullata*, Sc). Oyster shell fragments of the same species treated in Chlorox™ (Sodium hypochlorite) for one day (TCbe) and marble chips (Mc) were also used.

Another set of these five substrates were soaked in aged seawater for one week prior to the experiment (SCbe, SCbi, SSc, STCbe, SMc) to establish a microbial film which could also be a factor triggering settlement.
Thus, five different substrate types were tested:

First set composed of five unsoaked substrates:
1a. New *Crassostrea bilineata* (Röding, 1798) shell fragments (Cbi),
2a. New *Crassostrea belcheri* (Sowerby, 1871) shell fragments (Cbe),
3a. New *Saccostrea cucullata* (Born, 1778) shell fragments (Sc),
4a. New Cbe shell fragments treated with Sodium hypochlorite (TCbe),
5a. New Marble chips: diameter, 3-4 mm. (Mc)

Second set composed of five soaked substrates:
1b. New Cbi soaked in aged seawater for one week (SCbi),
2b. New Cbe soaked in aged seawater for one week (SCbe),
3b. New Sc soaked in aged seawater for one week (SSc),
4b. TCbe soaked in aged seawater for one week (STCbe),
5b. Mc soaked in aged seawater for one week (SMc)

Experimental design
The experiments were conducted in Nunclon® plastic tissue culture plates (diameter of each well was 3 cm). Aged sea water (30 ppt salinity) was mixed with distilled fresh water until a salinity of 18 ppt was reached, which is the ideal salinity for the hatching of *C. belcheri* and kept at room temperature (26-28 °C). Test treatments were conducted in two water types (aged sea water and conditioned aged seawater) and in two soaking conditions (unsoaked and soaked) on five substrate types (Cbi, Cbe, Sc, TCbe and Mc). Triplicate wells were prepared with each set of treatment on substrates. In each replicate, approximately 50-100 larvae were placed in a total volume of 8 ml of the tested water types. The number of settling larvae was recorded after 24 hours: the end of the experiment. The choice of 24 hours for data recording followed a preliminary observation with the highest mean settling frequency at 24 hours compared to 12 and 48 hours. Food was not provided during the experiment. Aged seawater was used to control of presence/absence of pheromones in combination with substrate. In all experimental treatments, the larvae were examined with a stereo microscope to determine the percentage of the total number of larvae that had set and cemented.

Analysis of data
There were three independent variables: two water types, five substrate types, two soaking conditions, and one dependent variable: settling frequency. Statistical analysis was conducted with the computer program JMP using 3-way ANOVA multifactorial analyses of water types, substrate types and soaking conditions. Interaction between three variables was first tested for significance. If a significant interaction was shown, it meant that some differences were significant among the

three studied variables. If not, then 2-way ANOVA and 1-way ANOVA at a 95 % level of significance was used for data analysis respectively (transforming the data of proportion percentage settling larvae to arcsine due to the non-normal distribution of high variance in recorded data). The value was back-transformed from arcsine to get the mean percentage.

RESULTS

1. Settling behaviour observation during attachment and cementing

Three main stages of settlement were observed. The first stage was free swimming searching, with the foot extended and the cilia of the velum beating. The second stage was the crawling pediveliger with its velum retracted, crawling smoothly with a gliding motion due to the pedal cilia only as in *Ostrea commercialis* (Roughley 1933). The foot then became shorter and broader, and contracted more often over the substrate. The pediveliger attached the tip of the foot to the substrate, dragging itself forward by contracting the foot. The larvae then re-extended the anterior portion of the foot preparing for the next forward movement. These contractions increased in violence with time, the foot becoming progressively more blunt, and the speed reduced progressively. The larvae always turned to the left, counterclockwise, by lifting the front half of the foot off the substrate and re-extending it in a new direction in a star shaped track over a small area. The third and final stage was cementing. It was initiated by a turn to the right, instead of the left as previously, by the lifting of the foot off the substrate and extending it in an opposite direction viz., to the right. This change characteristically marked the start of cementing. The shell valves of the larvae were pulled over the foot, and several sharp twitches of the shell followed. The larva then rolled over onto the left valve and the foot, attached by the tip, keeping it pressed against the substrate. The very broad foot remained protruding from the shell and held the larva to the substrate. After two minutes the foot was slowly withdrawn into the shell. During cementing the mantle fold vibrated but did not extend beyond the margins of the shell valves.

The foot was observed to be the organ of final attachment and the squeezing out of byssus substance on to the substrate could be followed. A byssus thread, probably of another nature, was observed during the late crawling stage and especially during the cementing stage.

2. Interaction between variables

The number of settling larval as a mean percentage at 24 hours in the two water types (aged sea water and conditioned aged sea water), two soaking conditions (unsoaked and soaked) and five substrate types (Cbi, Cbe, Sc, TCbe, and Mc) are shown in Table 1. Interaction was shown to be non-significant among three variables; water types, soaking conditions and substrate types (3-way ANOVA, df = 4, F = 0.1386, P = 0.9669). Two-way ANOVA showed no significant diffeence between

water types and soaking conditions (DF = 1, F = 0.7407, P = 0.3946) and between water types and substrate types (DF = 4, F = 1.2456, P = 0.3703). However, it showed a highly significant interaction between soaking conditions and substrate types (df = 4, F = 3.7857, P = 0.0106).

3. Influence of water type on settling

There was a highly significant difference in the settling frequency in the two water types, type A (conditioned aged sea water) and type B (aged sea water) (df = 1, F = 19.1103, P <0.0001). Almost twice the settling frequency was found in conditioned aged seawater over aged sea water (Fig. 1).

4. Influence of soaking

There is a highly significant difference in settling frequency between the soaking conditions, soaked and unsoaked (df = 1, F = 14.1663, P = 0.0005). The soaked substrates had about a 1.5 times higher settling frequency (Fig. 2). There was a significant effect on larval settling frequency under different soaking conditions related to substrate types (df = 4, F = 3.7857, P = 0.0106).

5. Influence of substrate type

There was a highly significant difference between the five substrate types (df = 4, F = 4.6017, P = 0.0038). The interaction was synergistic with soaked substrates always showing higher settling frequencies than unsoaked substrates of the same type (Fig. 3). Shell fragments (Cbe) of its own species induced highest settling frequencies in both unsoaked and soaked conditions (1.3 times increase) while the Cbi substrates were found to be favoured in second place. Different genus substrate, SSc, showed surprisingly an increase in settling frequency of about five times when soaked. TCbe and Mc showed a small increase in settling when soaked, with Mc more favoured by the larvae.

There were significant differences of settling frequency in paired comparisons between Cbi-Sc, Cbe-Sc, Cbe-TCbe, Cbi-SCbe, Cbi-SSc, Sc-SCbi, Sc-SCbe, Sc-SSc, Sc-SMc, TCbe-SCbi, TCbe-SCbe, TCbe-SSc, Mc-SCbe, Mc-SSc, SCbi-STCbe, SCbe- STCbe, SMc, SSc-STCbe, SSc-SMc) (df = 9, P<0.05). The substrates Cbi, Cbe and Mc showed statistically higher settling frequencies among unsoaked substrates (df = 1, P<0.05), while substrate Sc inducing the lowest settling frequency. Substrates SCbe and SSc showed statistically higher settlement among soaked substrates (df= 1, P<0.05) while the soaked substrates STCbe and SMc showed significantly low settlement.

Table 1. Oyster larvae of *Crassostrea belcheri* settling in 2 different water types, during 2 different soaking conditions and on 5 substrate types. Data is expressed as mean percentage of settling with standard deviation (3 replicates) at 24 hours, end of experiment.

Water types	Soaking conditions	Substrate types	Mean±SD
A). Age sea water	a. Unsoaked	1. *C. bilineata* (Cbi)	35.64±21.67
		2. *C. belcheri* (Cbe)	57.14±31.19
		3. *S. cucullata* (Sc*)*	5.16±5.82
		4. Treated Cbe (TCbe)	9.07±11.23
		5. Marble chips (Mc)	19.09±10.97
	b. Soaked	1. Soaked Cbi (SCbi)	56.16±21.30
		2. Soaked Cbe (SCbe)	82.73±23.36
		3. Soaked Sc (SSc)	78.00±34.70
		4. Soaked TCbe (STCbe)	9.311±4.99
		5. Soaked Mc (SMc)	39.00±25.52
B). Conditioned aged seawater	a. Unsoaked	1. *C. bilineata* (Cbi)	66.67±32.48
		2. *C. belcheri* (Cbe)	68.45±52.16
		3. *S. cucullata* (SSc)	26.47±20.12
		4. Treated Cbe (TCbe)	59.58±3.39
		5. Marble chips (Mc)	68.07±18.10
	b. Soaked	1. Soaked Cbi (SCbi)	85.22±8.51
		2. Soaked Cbe (SCbe)	89.80±4.90
		3. Soaked Sc (SSc)	91.11±3.85
		4. Soaked TCbe (STCbe)	62.28±29.48
		5. Soaked Mc (SMc)	59.35±34.99

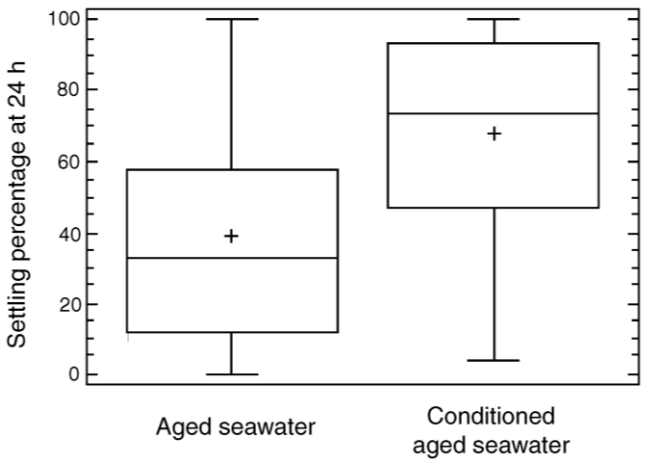

Figure 1. Mean percentage of *Crassostrea belcheri* larvae settling on conditioned aged sea water and aged seawater.

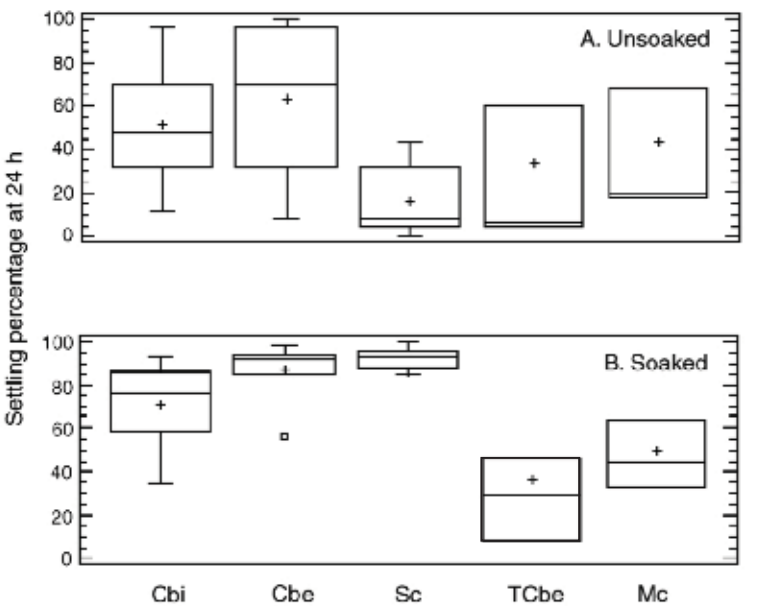

Figure 2. Mean percentage of *Crassostrea belcheri* larvae settling on A. 5 unsoaked substrates (Cbi = *Crassostrea bilineata*, Cbe = *C. belcheri*, Sc = *Saccostrea cucullata*, TCbe = treated *C. belcheri*, Mc = marble chips) and B. 5 soaked substrates.

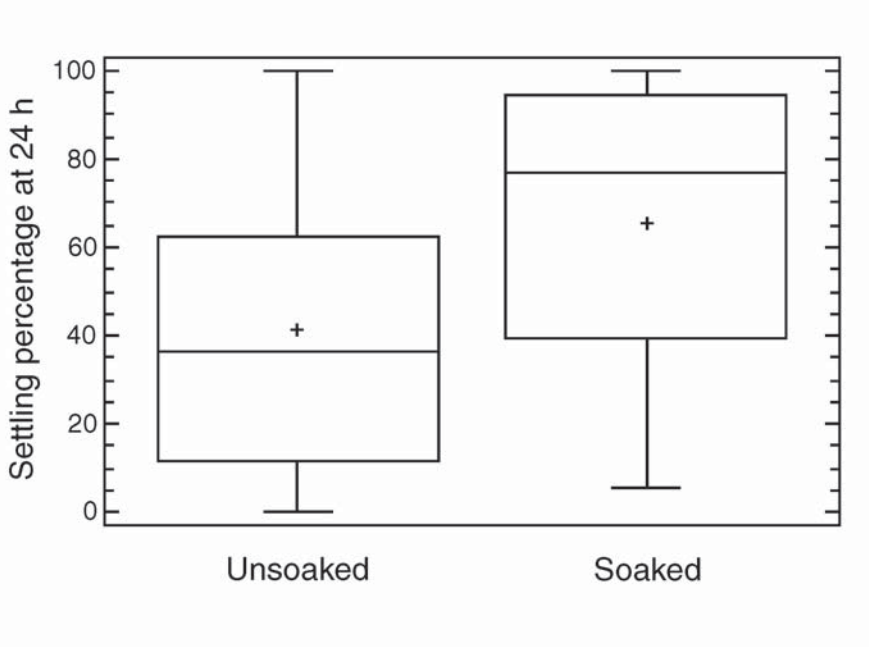

Figure 3. Mean percentage of *Crassostrea belcheri* larvae settling on unsoaked substrates and soaked substrates, respectively.

DISCUSSION

Competent pediveliger larvae find suitable substrates to attach as soon as possible for their successful settling and metamorphosis. However, it is essential for any species of oyster to keep a stage for substrate selection in their lives. Optimal settling time could be less than 24 hours in nature. In our experiments the larvae were not given phytoplankton during the 24 hour experiment to limit the number of external factors. Competent pediveliger larvae must be assumed to have energy sources.

The attachment behaviour of *C. belcheri* larvae was similar to that of *C. virginica* as observed by Nelson (1924) and Prytherch (1934) and that of *O. edulis* as observed by Cranfield (1973) and Cole & Knight-Jones (1939). These authors all noted three stages in settlement, except Cranfield (1973) who reported more details and distinguished between six phases. Turning of the larva to the right by 180 degrees and withdrawal of the foot was observed to be completed in about two minutes in *C. belcheri* and thus was different from the behaviour of *O. edulis.* The later species was found to turn only by 30 degrees and required 4-9 minutes for the withdrawal of the foot at the cementing stage (Nelson, 1924).

Our observations of the attachment of oyster larvae agreed with those of Stafford: 1910, (cited by Nelson, 1924). Both studies reported that the foot was the organ of final attachment where a large part of the base of foot of the full grown larvae

was developed into the byssus gland. The cells of this gland were gorged with a transparent secretion, which we did not find in the much shrunken gland which characterized the larva immediately after attachment. The extrusion of the mantle for the short period evidently aided in the quick and economical distribution of the cementing fluid as it was poured out of the byssus gland at the ventral edge of the left valve. This secretion hardened within less than 10 minutes. The circular movements of the larvae prior to final fixation not only aided them in finding a spot suitable for attachment, but this behaviour also tended to keep the larvae separated from each other.

Influence of water type

This study clearly shows that settlement frequencies are higher on all types of substrate soaked in water where adults were present prior to the experiment. This experiment adds evidence that oyster settling is initiated through the action of pheromones from the adults. The response to pheromones would account for the greater settlement on natural oyster shells in the presence of living spat and adults as observed by Cole and Knight-Jones (1949).

In this work, neither the chemical nature nor the concentration of the waterborne pheromone released by living adult oysters in the tested water was determined. However, Bayne (1969) demonstrated that pheromone inducing settlement extracted from oyster tissue of *O. edulis* was species-specific, was resistant to drying and boiling and was effective in promoting larval settlement in a concentration of 0.1 mg protein/ml. It would be interesting to study further the species-specific larval settlement inducted by different waterborne pheromones from tropical adult oysters such as the commercial species *C. belcheri* (Cbe) and *C. bilineata* (Cbi). An attempt was made to conduct settlement experiments on species specificity in different species of larvae related to waterborne pheromones, but the results were inconclusive because the *C. bilineata* larvae used were not in the right stage to settle at that time, due to a longer larval development of this species (17-21 days) than in *C. belcheri*.

Influence of soaking condition

Two groups of substrates were soaked for one week in aged seawater. We assumed that a thin bacterial film that larvae of *C. belcheri* prefer to settle on would coat their surface. The importance of such a bacterial film for the induction of settlement was demonstrated for *O. edulis* by Cole and Knight Jones (1949). This suggests that oyster farmers should soak the cultches for a greater spat fall.

The larval settlement on soaked shell fragments of *S. cucullata* (SSc) showed a great difference when compared to unsoaked substrate of that species ($P < 0.05$). This could be explained by the influence of a bacterial film. It would increase the settling on this species of shells and explain the great gregariousness in natural habitats of this species.

Influence of substrate type

The higher degree of settlement on its own species (Cbe) could be due either to the recognition of shell periostracum protein, conchiolin (Cbe) or to pheromones from adults (Cbe) and could explain the gregariousness of this species in nature. The destruction of the organic layers on the surface (TCbe) clearly rendered it a substrate unfavourable to the larvae settling on shells of its own species even after soaking (STCbe), where the effect of the bacterial film on the surface could be expected to stimulate an increase of larvae settling as observed in *C. virginica* by Crisp (1967). The different species of shell substrate of the same genus (Cbi) were rather favoured by the larvae of *C. belcheri* (Cbe) contrary to substrate of a different genus (Sc).

CONCLUSIONS

1. The settling behaviour of *Crassostrea belcheri* has been described. The pediveliger larva when competent for settling swims with the foot more or less protruding between the valves, adjacent to the velum. When coming into contact with solid substrates, it adheres and crawls by means of the foot, first smoothly and later with jerky movements. When about to set the larva turns to the left and cirles over a very small area. Finally, it turns to the right and comes to rest and rests with the left valve undermost. During the final stage of settling (cementing) the foot of the larvae changes in shape becoming shorter, broader, and stouter. Fixation is effected by cement secreted from the byssus gland located in the foot.
2. The presence of adult oysters stimulates the settling of larvae of the same species. This suggests the effect of pheromones released from the adults.
3. There are significant differences between substrate types preferred by oyster larvae for settlement. Substrates soaked in seawater conditioned by the presence of adults had a higher degree of larval settlement.
4. Shell fragments of the same species had a higher degree of settlement than fragments from another species of the same genus. The lowest degree of settlement was found on shell fragments from a different genus.

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