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Attachment, metamorphosis and early growth of larval abalone *Haliotis diversicolor supertexta* in response to *Cocconeis placentula* var. *euglypta* and mixed diatoms

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ABSTRACT

The attachment, metamorphosis and growth response of larval abalone Haliotis diversicolor supertexta to a benthic diatom Cocconeis placentula var. euglypta and mixed diatoms were investigated in the laboratory. Larval attachment and metamorphosis were significantly higher on well-grown diatom species C. placentula var. euglypta than on mixed diatoms and the blank control. Better growth of newly attached abalone larvae was also observed on the C. placentula var. euglypta during the early rearing days. All the results indicated that larval attachment in response to diatom films depended either on diatom species, or the diatom cell density, or the age of diatom film, or the physiological condition of diatom. High larval attachment on the diatom films yielded high larval metamorphosis, survival and shell length. The early growth of abalone larvae also depended on the diatom films.

KEYWORDS

Abalone;
Attachment;
Metamorphosis;
Diatom.

INTRODUCTION

Abalone is an economically important kind of gastropods in many countries[1]. China is one of the earliest countries that utilize the abalone. The culture of abalone has made great advances in China. Presently, the annual output of commercial abalone has surmounted 2000 tons[2].

Diatoms are important natural resources for abalone farm, especially in seed production. Benthic diatoms are commonly used as settlement cues and principal food for larval abalone[3,4]. Many researches have been studying on the relationship between diatoms and abalone larvae, so techniques for abalone seed production are developed continually. However, it has also been shown that responses to diatoms may be species-specific[5,6]. As a temperate water species, *H. diversicolor supertexta* was cultured popularly in South China, including Taiwan. But mortalities of larval abalone during the nursery rearing often occurred in these areas. To find out the reasons, more data about the relationship between diatoms and the larval abalone *H. diversicolor supertexta* need to be studied.

In this study, we investigated the effectiveness of *C. placentula* var. *euglypta* and mixed diatoms on larval settlement (attachment and metamorphosis) and early

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growth of larval abalone *H. diversicolor supertexta*. We also examined whether the physiological condition and density of the diatom film have an effect on the abalone larvae.

MATERIALS AND METHODS

Abalone larvae

Experiments were conducted in Dongshan, Fujian Province of China from September to October, 2003. Larvae of *H. diversicolor supertexta* were obtained from a local abalone farm. The trochophore larvae were transported to the laboratory in 1.5h and reared in 1μm filtered natural seawater (FSW) at 25°C. Competent larvae were selected at night to be used in the experiments after the larval density was estimated[5].

Diatoms

Cocconeis. placentula var. *euglypta*, cell length 20~48μm and width 12~25μm, was isolated from the naturally growing diatoms on plastic sheets used in the abalone seed production. Mixed diatoms were collected from abalone farms in Dongshan, Fujian and Nan'ao, Shenzhen respectively. Diatoms were attached to glass slides (76.2mm×25.4mm×1mm) and cultured in f/2 medium[7] at 25°C and with a 12h L: 12h D illuminance cycle. Diatoms were treated as TABLE 1 showed before they were used in the experiments.

H_2O_2 treatment was used to obtain different physiological condition of *Cocconeis placentula* var. *euglypta* in the experiments. SD2 and SD4 were treated with H_2O_2 (30ppm) for 48h and soaked for three times (two hours every time) to remove the H_2O_2 residuals farthest before they were used in the experiments. Physiological condition of diatoms was evaluated according to the color of chromatophore. After H_2O_2 treatment, the chromatophore of these diatoms was reduced or even disappeared in color for the strong oxidation of H_2O_2.

Initial diatom cell density and species composition on glass slides were measured immediately before the experiments. Mixed diatoms from Nan'ao are composed mainly of *Cocconeis* spp. (dominated by *C. placentula* var. *euglypta*, 33.19±5.91%), *Navicula* spp. (29.97±0.69%), *Amphora* spp. (19.70 ±6.39%), *Melosira moniliformis* (7.64±1.30%), *Asteroplanus karianus* (6.98±1.94%), *Achnanthes* spp. (0.96±0.27%), *Amphiprora alata* (0.78 ±0.28%), *Nitzschia* spp. (0.78±0.288%). Mixed diatoms from Dongshan are composed mainly of *Cocconeis* spp. (including *C. placentula* var. *euglypta*, 27.58±1.9%), *Navicula* spp. (57.25 ±3.05%), *Nitzschia* spp. (12.74±2.51%), *Amphora* spp. (2.43±1.10%).

TABLE 1 : Diatoms used in settlement and growth experiments

Substratum*	Diatom (attached on glass slides)	Density (cell cm^{-2}, mean ± se.)	Culture Time (days)	H_2O_2 treatment	Growth condition
Experiment 1					
SD1	*Cocconeis placentula* var. *euglypta*	$(7.01 \pm 0.82) \times 10^5$	12	not treated	well
SD2	*Cocconeis placentula* var. *euglypta*	$(9.29 \pm 0.29) \times 10^5$	12	treated	bad
MD1	Mixed diatoms from Nan'ao	$(1.22 \pm 0.12) \times 10^3$	5	not treated	young
MD2	Mixed diatoms from Nan'ao	$(5.56 \pm 1.48) \times 10^5$	29	not treated	aged
Control	/	/	/	/	/
Experiment 2					
SD3	*Cocconeis placentula* var. *euglypta*	$(8.45 \pm 0.56) \times 10^5$	14	not treated	well
SD4	*Cocconeis placentula* var. *euglypta*	$(13.20 \pm 1.5) \times 10^5$	14	treated	bad
MD3	Mixed diatoms from Nan'ao	$(4.63 \pm 1.13) \times 10^4$	14	not treated	well
MD4	Mixed diatoms from Nan'ao	$(9.78 \pm 0.60) \times 10^2$	14	not treated	well
Control	/	/	/	/	/

***SD2 and SD4 were treated with H_2O_2 (30ppm) for 48h before they were used in the experiments. The chromatophore of these diatoms was reduced or even disappeared in color. SD1 and SD3 were untreated with H_2O_2 and well in growth. For culturing in f/2 medium for more than 4 weeks, the diatoms of MD2 were aging and parts were sloughing off. MD1 was cultured for only 5 days, so the diatom film was very young and the cell density was low. Diatom density of MD3 was significantly higher than MD4 ($p<0.05$). The low cell density of MD4 was mainly due to the low cell density of algal inoculation.**

Settlement and growth experiments

Three glass tanks (44cm×23cm×30cm) were prepared as three replicates for the experiments. Two filmed slides of each treatment and controls without any diatoms (TABLE 1) were randomly placed on the bottom of each tank, containing 10L of FSW. One air stone of each tank and a 12h L: 12h D illuminance cycle were provided. Temperature and salinity were 25±1°C and 31gL^{-1} respectively. Approximately 36h after fertilization, the larvae were added to each tank at 30 individuals per liter in experiment 1 and 400 individuals per liter in experiment 2. The density of abalone larvae was estimated by counting the larvae in ten 10-ml subsamples drawn from the whole population[8].

Here we considered the day when the eggs were fertilized as the first day of the experiments and accordingly, the larvae were one day old. The number of attached, metamorphosed and dead larvae was counted under optical microscope when the larvae were 4, 8 and 5, 9 days old in each experiment. At the same time, the microscope with an ocular graticule was used to measure the shell length (SL) of individuals which were selected on random[5]. Attached larvae were considered as those which displayed firm attachment of their foot to the substratum. Post-larvae (metamorphosed larvae) were those which lost their cilia and began formation of the juvenile shell[9]. Dead larvae were those which only had empty cuticle or were deteriorated in the outer cuticle and inactivity. The slides were removed from the tanks after they were checked.

Data analysis

T-test was used to determine significant differences of the diatom density. The treatments were not independent because the attractiveness of substrate changes as soon as there were attached conspecific larvae present. Consequently, data from the larval 'choice' experiments were analysed using paired t-tests[8]. Correlation analysis was used to determine the correlation between the larval attachment, metamorphosis and survival and growth.

RESULTS

Significantly higher number of larvae attached on untreated *C. placentu*la var. *euglypta* (SD1 or SD3) than on treated conspecific diatom (SD2 or SD4) and other substrata (for each, $p<0.05$, TABLE 2) in each experiment. In experiment 1, no significant differences were observed among the young mixed diatoms from Nan'ao (MD1), the aged mixed diatoms from Nan'ao (MD2) and the blank control (for each, $p>0.05$, TABLE 2) on Day 4, but more larvae attached on MD1 than on MD2 and the control. In experiment 2, the number of attached larvae was significantly higher on high-density mixed diatoms from Dongshan (MD3) compared with low-density mixed diatoms from Dongshan (MD4) on Day 5 ($p<0.05$, TABLE 2).

Similar to the larval attachment, both experiments showed significantly higher number of larvae complete metamorphosis on SD1 and SD3 compared respectively with other diatom films and the blank control (for each, $p<0.05$, TABLE 3). In experiment 1, no larvae were observed to complete metamorphosis on MD2 and the control when larvae were 4 days old (TABLE 3). In experiment 2, significantly more larvae completed metamorphosis on MD3 compared with SD4, MD4 and the control (for each, $p<0.05$, for control, $p<0.01$; TABLE 3) when larvae were 5 days old. All the surviving larvae completed metamorphosis on Day 8 in experiment 1 and on Day 9 in experiment 2.

TABLE 2 : The number of attached larval abalone *H. diversicolor supertexta* on each substrate*

Experiment	Time (day)	Number of attached larvae				
		SD1	**SD2**	**MD1**	**MD2**	**Control**
Exp. 1	8	28.76 ± 7.57^{c}	10.67 ± 1.53^{b}	4.67 ± 2.08^{a}	3.33 ± 1.05^{a}	2.67 ± 1.53^{a}
		SD3	**SD4**	**MD3**	**MD4**	**Control**
Exp. 2	9	322.00 ± 73.18^{c}	36.67 ± 10.60^{ab}	101.67 ± 27.02^{b}	37.67 ± 8.62^{ab}	4.00 ± 2.65^{a}

***SD1 and SD3 were *C. placentula* var. *euglypta* untreated with H_2O_2. SD2 and SD4 were *C. placentula* var. *euglypta* treated by H_2O_2. MD1 and MD2 were mixed diatoms from Nan'ao, and MD3 and MD4 were mixed diatoms from Dongshan. MD1 and MD4 were low in cell density, and MD2 was aged diatoms.**

Values in the same row sharing a common superscript letter are not significantly different ($p>0.05$).

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TABLE 3 : The number of metamorphosed larval abalone *H. diversicolor supertexta* on each substrate*

Experiment	Time (day)	Number of attached larvae				
		SD1	SD2	MD1	MD2	Control
Exp. 1	8	19.33 ± 4.04^{c}	5.67 ± 2.520^{b}	0.67 ± 0.58^{a}	0	0
		SD3	SD4	MD3	MD4	Control
Exp. 2	9	280.00 ± 69.78^{c}	22.67 ± 8.50^{bc}	81.33 ± 29.57^{b}	13.33 ± 4.04^{ab}	1.67 ± 1.15^{a}

***SD1 and SD3 were *C. placentula* var. *euglypta* untreated with H_2O_2. SD2 and SD4 were *C. placentula* var. *euglypta* treated by H_2O_2. MD1 and MD2 were mixed diatoms from Nan'ao, and MD3 and MD4 were mixed diatoms from Dongshan. MD1 and MD4 were low in cell density, and MD2 was aged diatoms.**

Values in the same row sharing a common superscript letter are not significantly different (p>0.05).

The number of surviving post-larvae was significantly higher on SD1 and SD3 compared respectively with other substrata in the two experiments (for each, p<0.05, TABLE 4). In experiment 1, significantly higher number of surviving post-larvae on SD2 and MD1 was observed than on MD2 on Day 8 (for each, p>0.05, TABLE 4). In experiment 2, significantly more post-larvae survived on MD3 than on SD4 (p<0.05, TABLE 4) both on Day 5 and 9. It was also noticed that the number of surviving larvae on each diatom film decreased significantly due to post-larvae mortality on Day 9 (TABLE 4).

In experiment 1, there was significant difference in mean shell length (SL) of 8-day-old post-larvae on SD1 and SD2 (p<0.05, TABLE 5). The mean SL of the post-larvae on SD1 was also significantly higher than on MD1, MD2 and the control on Day 8 (for each, p<0.05, TABLE 5). In experiment 2, mean SL of the post-larvae on SD3 was significantly higher than on SD4, and MD3 was higher than MD4 on Day 9 (for each, p<0.05, TABLE 5). TABLE 6 showed the higher larval growth rate on SD1, SD3 and MD3 compared respectively with other substrata in the two experiments (for each, p<0.05, TABLE 6).

It showed that there was positive correlation between the numbers of attached larvae with metamorphosed or survival post-larvae, according to the correlation analysis (TABLE 7). The correlation analysis also showed the positive correlation between the growth of larvae and the metamorphosis of larvae and the survival

TABLE 4 : The number of surviving post-larval abalone *H. diversicolor supertexta* on each substrate*

Experiment	Time (day)	Number of attached larvae				
		SD1	SD2	MD1	MD2	Control
Exp. 1	8	23.67 ± 9.07^{c}	7.00 ± 4.00^{b}	4.00 ± 1.00^{b}	1.67 ± 1.15^{a}	1.00^{a}
		SD3	SD4	MD3	MD4	Control
Exp. 2	9	87.67 ± 23.03^{bc}	5.00 ± 2.65^{b}	18.33 ± 6.03^{ab}	4.00 ± 1.73^{b}	2.33 ± 1.15^{a}

***SD1 and SD3 were *C. placentula* var. *euglypta* untreated with H_2O_2. SD2 and SD4 were *C. placentula* var. *euglypta* treated by H_2O_2. MD1 and MD2 were mixed diatoms from Nan'ao, and MD3 and MD4 were mixed diatoms from Dongshan. MD1 and MD4 were low in cell density, and MD2 was aged diatoms.**

Values in the same row sharing a common superscript letter are not significantly different (p>0.05).

TABLE 5 : The shell length* of post-larval abalone *H. diversicolor supertexta* on each substrate

Experiment	Time (day)	Shell length (μm, mean ± s.e.)				
		SD1	SD2	MD1	MD2	Control
Exp. 1	8	410.83 ± 48.31^{c}	364.17 ± 32.46^{b}	346.25 ± 19.59^{b}	353.57 ± 24.70^{b}	314.38 ± 64.04^{b}
		SD3	SD4	MD3	MD4	Control
Exp. 2	9	439.17 ± 36.74^{c}	375.00 ± 44.77^{b}	423.33 ± 41.87^{ab}	363.64 ± 39.71^{b}	347.17 ± 38.21^{a}

***The shell length was measured as the most crow-fly distance between the rear and front of the larva under optical microscope with an ocular graticule.**

***SD1 and SD3 were *C. placentula* var. *euglypta* untreated with H2O2. SD2 and SD4 were *C. placentula* var. *euglypta* treated by H2O2. MD1 and MD2 were mixed diatoms from Nan'ao, and MD3 and MD4 were mixed diatoms from Dongshan. MD1 and MD4 were low in cell density, and MD2 was aged diatoms.**

Values in the same row sharing a common superscript letter are not significantly different (p>0.05).

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TABLE 6 : The growth rate of larvae abalone *H. diversicolor supertexta* on each substrate*

Experiment	Time interval (day)	Growth rate (μm/d, mean ± s.e.)				
		SD1	SD2	MD1	MD2	Control
Exp. 1	4-8 (5 days)	26.79 ± 5.96^{c}	19.50 ± 4.52^{b}	21.59 ± 1.13^{b}	21.89 ± 1.66^{b}	14.72 ± 9.38^{b}
		SD3	SD4	MD3	MD4	Control
Exp. 2	5-9 (5 days)	23.74 ± 0.87^{c}	17.83 ± 4.66^{b}	25.10 ± 0.70^{c}	17.70 ± 4.74^{b}	17.01 ± 3.54^{b}

***SD1 and SD3 were *C. placentula* var. *euglypta* untreated with H_2O_2. SD2 and SD4 were *C. placentula* var. *euglypta* treated by H_2O_2. MD1 and MD2 were mixed diatoms from Nan'ao, and MD3 and MD4 were mixed diatoms from Dongshan. MD1 and MD4 were low in cell density, and MD2 was aged diatoms.**

Values in the same row sharing a common superscript letter are not significantly different (p>0.05).

TABLE 7 : Correlation analysis on the number of attached, metamorphosed and survival larvae in experiment 1 and 2

	Experiment	Correlation coefficient
The number of attached larvae	Exp. 1	0.999
and metamorphosed larvae	Exp. 2	0.998
The number of attached larvae	Exp. 1	0.997
and survival post-larvae	Exp. 2	0.993
The number of metamorphosed larvae	Exp. 1	0.909
and shell length of post-larvae	Exp. 2	0.862
The number of metamorphosed larvae	Exp. 1	0.735
and growth rate of larvae	Exp. 2	0.713
The number of survival larvae	Exp. 1	0.918
and shell length of post-larvae	Exp. 2	0.810
The number of survival larvae	Exp. 1	0.783
and growth rate of larvae	Exp. 2	0.649

TABLE 8 : Percentage of attached and metamorphosed larvae on each block of treatments of all larvae*

Experiment	Time (day)	% of attached larvae on each block of treatments (mean ± se)
Exp. 1	4	16.67 ± 2.19
	8	21.67 ± 4.58
Exp. 1	5	12.55 ± 1.97
	9	8.90 ± 1.26
		% of metamorphosed larvae on each block of treatments (mean ± se)
Exp. 1	4	8.55 ± 0.77
	8	12.44 ± 3.42
Exp. 1	5	9.98 ± 1.77
	9	2.93 ± 0.46

***% of attached or metamorphosed larvae on each block of treatments = total number of attached or metamorphosed larvae on each block of treatments/total number of larvae in the whole glass tank.**

of post-larvae (TABLE 7). The percentage of attached and metamorphosed larvae on each block of treatments in experiment 1both increased on Day 8, while they decreased in experiment 2 on Day 9 (TABLE 8).

DISCUSSION

The significant higher larval attachment on untreated *C. placentula* var. *euglypta* than mixed diatoms shows that it is one of suitable species as attachment substratum for *H. diversicolor supertexta* may because of their uniform cells and prostrate type of cell growth[10]. In addition, the presence of uneven diatom species in mixed diatoms such as *Melosira moniliformis*, *Asterionella kariana*, *Achnanthes* spp. and *Amphiprora alata* may be an added factor that prevented successful attachment of larvae. Lower larval attachment on mixed diatom MD1, MD2 and MD4 may be due to lower cell density of MD1 and MD4, and the too old age of MD2. The oxygen level on the aged diatom films maybe unstable as other study showed[11], so it may influence the settlement and early survival of the larvae. In experiment 2, the higher density of mixed diatom MD3 brought out significantly higher larval attachment than MD4. Kawamura and Kikuchi (1992) also reported that larvae of *H. discus hannai* preferred to attach on films with high diatom density. All these indicated the importance of the amount of diatom or the age of diatom film for larval attachment. Furthermore, higher larval attachment on *C. placentula* var. *euglypta* than on the same species treated with H2O2 showed that larval attachment also depends on the physiological conditions of diatoms.

Differences between shell lengths of the post-larvae on each diatom film might be due to the different quantity and quality of extracellular substances secreted by diatoms. In our experiments, the extracellular substances secreted by well-grown *C. placentula* var.

euglypta appeared to be sufficient for the larval metamorphosis and early growth because of its high cell density and healthy cell condition, though *Cocconeis* is considered poor in mucus secretion[1]. The low cell density of mixed diatom MD1 and MD4 might have small amount of extracellular substances, and consequently resulted in relatively low larval metamorphosis and shell length. Low shell length on aged mixed diatom MD2 may be due to the instability of the diatom film[5] and the decreasing amount of extracellular substances excreted by diatoms as a result of cell death and/or bacterial utilization[12,13]. Up to the present, little data have been published about the role of extracellular substances of diatoms in the early development of abalone larvae. Their structure and functions, therefore, need to be identified further.

Results of correlation analysis (TABLE 7) showed that higher larval attachment brought out higher larval metamorphosis, survival and shell length. This might be explained by the low abilities of abalone larvae to move around and search for better films after attachment, possible benefits of residual yolk and exogenous organic material, and the inability to digest diatom cell contents at very young age[14,15]. This finding is similar to the study of Daume et al. (1999a), who found that high settlement occured on *Sporolithon durum* (red alga) and *Navicula ramosissima*, and both settlement substrata yielded the highest growth rates and survival of abalone larvae *H. laevigata*.

In conclusion, diatom film together with its secretion plays a critical role in early development of abalone larvae, thus selecting appropriate substrata becomes important for abalone larvae and may decide the future fate of larvae. In practice, maintaining a suitable diatom film is critical in the success of abalone seed production[16]. In experiment 2, we found that the number of surviving larvae decreased significantly ($p<0.05$) on Day 9. It was possibly because too abalone larvae attached on the substrata, and consequently there might be short of oxygen and/or nutrition needed by larvae[17]. So maintaining appropriate amount of larvae in culture system is also important.

CONCLUSIONS

Our experiments has shown that well-grown diatom species *C. placentula* var. *euglypta* can bring out higher settlement and better early-growth of larval abalone *H. diversicolor supertexta*. Larval attachment in response to diatom films depends on diatom species, the cell density or age of diatom film and the physiological condition of diatom. The extracellular substances are important as well since their production relates to the cell density and growth condition of diatom film. High larval attachment can bright out high larval metamorphosis, survival and better early-growth of larval abalone *H. diversicolor supertexta*. The extracellular substances seemed to play an important role in larval metamorphosis or early growth of the abalone, which need further research.

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